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TITLE OF THE INVENTION (280 characters max)									
REGULATION OF HUMAN PHOSPHOLIPASE-LIKE ENZYME									
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☒ NO☐ YES, the name of the U.S. Government agency and the Government contract number are: _____

Respectfully submitted,

SIGNATURE Lisa M. Hemmendinger DATE October 10, 2000TYPED or PRINTED NAME Lisa M. Hemmendinger REG. NO. (if appropriate) 42,653☐ Additional inventors are being named on separately numbered sheets attached hereto**PROVISIONAL APPLICATION FILING ONLY**

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REGULATION OF HUMAN PHOSPHOLIPASE-LIKE ENZYME

TECHNICAL FIELD OF THE INVENTION

The invention relates to the area of enzyme regulation. More particularly, the invention relates to the regulation of human phospholipase-like enzyme and its regulation.

BACKGROUND OF THE INVENTION

The secretory phospholipase A2 (PLA2) superfamily comprises a number of heterogeneous enzymes whose common feature is to hydrolyze the sn-2 fatty acid acyl ester bond of phosphoglycerides. U.S. Patent No. 6,103,469. Hydrolysis of the glycerophospholipids releases free fatty acids and lysophospholipids. PLA2 activity generates precursors for the biosynthesis of biologically active lipids, hydroxy fatty acids, and platelet-activating factor.

PLA2 hydrolysis of the sn-2 ester bond in phospholipids generates free fatty acids, such as arachidonic acid and lysophospholipids. Arachidonic acid is processed into bioactive lipid mediators of inflammation, such as lyso-platelet-activating factor and eicosanoids. The synthesis of arachidonic acid from membrane phospholipids is the rate-limiting step in the biosynthesis of the four major classes of eicosanoids (prostaglandins, prostacyclins, thromboxanes and leukotrienes) involved in pain, fever, and inflammation. Furthermore, leukotriene-B4 is known to function in a feedback loop which further increases PLA2 activity. Wijkander *et al.*, *J. Biol. Chem.* 270, 26543-49, 1995.

PLA2s, first described as components of snake venoms and pancreatic juice and later defined in mammals, have traditionally been classified into several major groups and subgroups based on their amino acid sequence, disulfide bond location, and divalent cation

requirements. The PLA2s of groups I, II, and III consist of low molecular weight, secreted, Ca^{2+} -dependent proteins, Group IV PLA2s are primarily 85-kDa, Ca^{2+} -dependent cytosolic phospholipases, and an additional group comprises the Ca^{2+} -independent cytosolic phospholipases. Davidson & Dennis, *J. Mol. Evol.* 31, 228-38, 1990; Dennis, *J. Biol. Chem.* 269, 13057-13060, 1994.

The first PLA2s to be extensively characterized were the Group I, II, and III PLA2s found in snake and bee venoms. These venom PLA2s share many features with mammalian PLA2s, including a common catalytic mechanism, the same Ca^{+} requirement, and conserved primary and tertiary structures. In addition to their role in the digestion of prey, the venom PLA2s display neurotoxic, myotoxic, anticoagulant, and proinflammatory effects in mammalian tissues. This diversity of pathophysiological effects is due to the presence of specific, high affinity receptors for these enzymes on various cells and tissues. Lambeau *et al.*, *J. Biol. Chem.* 270, 5534-40, 1995.

At least four different groups of PLA2s have been characterized in mammalian cells, including Group I (pancreatic), Groups IIA, and IIC (inflammatory), and Group V (expressed in the heart). The pancreatic PLA2s function in the digestion of dietary lipids and have been proposed to play a role in cell proliferation, smooth muscle contraction, and acute lung injury. The inflammatory PLA2s are potent mediators of inflammatory processes and are highly expressed in serum and synovial fluids of patients with inflammatory disorders. These Group II PLA2s are found in most human cell types assayed and are expressed in diverse pathological processes such as septic shock, intestinal cancers, rheumatoid arthritis, and epidermal hyperplasia. A Group V PLA2 has been cloned from brain tissue and is strongly expressed in heart tissue. Other PLA2s have been cloned from various human tissues and cell lines, suggesting a large diversity of PLA2s. A human PLA2 was recently cloned from fetal lung, and based on its structural properties, appears to be the first member of a new group of mammalian PLAs, referred to as Group X. Chen *et al.*, *J. Biol. Chem.* 269, 2365-68, 1994; Kennedy *et al.*, *J. Biol. Chem.* 270, 22378-85, 1995; Komada *et al.*, *Biochem. Biophys. Res. Commun.* 168, 1059-65, 1990; and Cupillard *et al.*, *J. Biol. Chem.* 272, 15745-52, 1997).

The discovery of a new human phospholipase A2 protein and the polynucleotides encoding it satisfies a need in the art by providing new compositions which are useful in the diagnosis, prevention, and treatment of cancer, inflammation, CNS disorders, cardiovascular disorders, chronic obstructive pulmonary disease, and obesity.

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SUMMARY OF THE INVENTION

It is an object of the invention to provide reagents and methods of regulating a human phospholipase-like enzyme. This and other objects of the invention are provided by one or more of the embodiments described below.

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One embodiment of the invention is a method of screening for agents which can regulate the activity of a human phospholipase-like enzyme. A test compound is contacted with a polypeptide comprising an amino acid sequence which is at least about 78% identical to the amino acid sequence shown in SEQ ID NO:2. Binding of the test compound to the polypeptide is detected. A test compound which binds to the polypeptide is thereby identified as a potential therapeutic agent for regulating activity of the human phospholipase-like enzyme.

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Another embodiment of the invention is a method of screening for agents which regulate an activity of a human phospholipase-like enzyme. A test compound is contacted with a polypeptide comprising an amino acid sequence which is at least about 78% identical to the amino acid sequence shown in SEQ ID NO:2. A phospholipase activity of the polypeptide is detected. A test compound which decreases the phospholipase activity is thereby identified as a potential therapeutic agent for decreasing the activity of the human phospholipase-like enzyme. A test compound which increases the phospholipase activity of the polypeptide is thereby identified as a potential therapeutic agent for increasing the activity of the human phospholipase-like enzyme.

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Yet another embodiment of the invention is a method of screening for agents which regulate an activity of a human phospholipase-like enzyme. A test compound is contacted with a product encoded by a polynucleotide which comprises a nucleotide sequence which is at least 50% identical to the nucleotide sequence shown in SEQ ID NO:1. Binding of the

test compound to the product is detected. A test compound which binds to the product is thereby identified as a potential therapeutic agent for regulating the activity of the human phospholipase-like enzyme.

Even another embodiment of the invention is a method of reducing activity of a human phospholipase-like enzyme. A cell is contacted with a reagent which specifically binds to a product encoded by a polynucleotide comprising a nucleotide sequence which is at least 50% identical to the nucleotide sequence shown in SEQ ID NO:1. The activity of the human phospholipase-like enzyme is thereby reduced.

Another embodiment of the invention is a pharmaceutical composition comprising a reagent which specifically binds to a product encoded by a polynucleotide comprising a nucleotide sequence which is at least 50% identical to the nucleotide sequence shown in SEQ ID NO:1 and a pharmaceutically acceptable carrier.

Another embodiment of the invention is a pharmaceutical composition comprising an expression construct encoding a polypeptide comprising the amino acid sequence shown in SEQ ID NO:2 and a pharmaceutically acceptable carrier.

The invention thus provides a human phospholipase-like enzyme which can be used to identify test compounds which may act, for example, as agonists or antagonists at the enzyme's active site. Human phospholipase-like enzyme and fragments thereof also are useful in raising specific antibodies which can block the enzyme and effectively reduce its activity.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1. BLASTP alignment of human phospholipase-like enzyme (SEQ ID NO:2) with the protein identified with SwissProt Accession No. Q05017 (SEQ ID NO:3).

FIG. 2. Prosite search results.

FIG. 3. HMMPFAM alignment of SEQ ID NO:2 with pfam|hmm|Lipase_GDSL.

FIG. 4. HMMPFAM alignment of SEQ ID NO:2 with pfam|hmm|Lipase_GDSL.

FIG. 5. HMMPFAM alignment of SEQ ID NO:2 with pfam|hmm|Lipase_GDSL.

DETAILED DESCRIPTION OF THE INVENTION

A novel human phospholipase-like enzyme is a discovery of the present invention. Human phospholipase-like enzyme comprises the amino acid sequence shown in SEQ ID NO:2. A coding sequence for human phospholipase-like enzyme is shown in SEQ ID NO:1. This coding sequence is found in GENBANK|AC074011.2 (genomic, chromosome 2) and GENBANK|AC022076.10 (partial genomic sequence on chromosome 3); this gene is duplicated on chromosome 2 and 3. Related ESTs (SEQ ID NOS:4-7) are expressed in squamous cell carcinomas, pooled human melanocyte, fetal heart, pregnant uterus, and adult colon.

Human phospholipase-like enzyme is 77% identical over 1217 amino acids to the rabbit protein identified with SwissProt Accession No. Q05017 and annotated as "PHOSPHOLIPASE ADRAB-B PRECURSOR (EC 3.1.-.-)" (FIG. 1).

By inference from sequence similarities, human phospholipase-like enzyme belongs to the "GDSL" family of lipolytic enzymes and has both esterase and phospholipase A/lysopholipase activities. Human phospholipase-like enzyme of the invention is expected to be useful for the same purposes as previously identified phospholipase enzymes. Human phospholipase-like enzyme is believed to be useful in therapeutic methods to treat disorders such as cancer, inflammation, CNS disorders, cardiovascular disorders, chronic obstructive pulmonary disease, and obesity. Human phospholipase-like enzyme also can be used to screen for human phospholipase-like enzyme agonists and antagonists.

Polypeptides

Human phospholipase-like enzyme polypeptides according to the invention comprise at least 6, 10, 15, 20, 25, 50, 75, 100, 125, 150, 175, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000, 1050, 1100, 1150, 1200, or 1210 contiguous amino acids selected from the amino acid sequence shown in SEQ ID NO:2 or a biologically active variant thereof, as defined below. A phospholipase-like enzyme

polypeptide of the invention therefore can be a portion of a phospholipase-like enzyme protein, a full-length phospholipase-like enzyme protein, or a fusion protein comprising all or a portion of a phospholipase-like enzyme protein.

Biologically Active Variants

Human phospholipase-like enzyme polypeptide variants which are biologically active, *e.g.*, retain a phospholipase or esterase activity, also are phospholipase-like enzyme polypeptides. Preferably, naturally or non-naturally occurring phospholipase-like enzyme polypeptide variants have amino acid sequences which are at least about 77, 80, 85, 90, 96, 96, or 98% identical to the amino acid sequence shown in SEQ ID NO:2 or a fragment thereof. Percent identity between a putative phospholipase-like enzyme polypeptide variant and an amino acid sequence of SEQ ID NO:2 is determined using the Blast2 alignment program (Blosum62, Expect 10, standard genetic codes).

Variations in percent identity can be due, for example, to amino acid substitutions, insertions, or deletions. Amino acid substitutions are defined as one for one amino acid replacements. They are conservative in nature when the substituted amino acid has similar structural and/or chemical properties. Examples of conservative replacements are substitution of a leucine with an isoleucine or valine, an aspartate with a glutamate, or a threonine with a serine.

Amino acid insertions or deletions are changes to or within an amino acid sequence. They typically fall in the range of about 1 to 5 amino acids. Guidance in determining which amino acid residues can be substituted, inserted, or deleted without abolishing biological or immunological activity of a phospholipase-like enzyme polypeptide can be found using computer programs well known in the art, such as DNASTAR software. Whether an amino acid change results in a biologically active phospholipase-like enzyme polypeptide can readily be determined by assaying for phospholipase activity, as described for example, in Example 4.

Fusion Proteins

Fusion proteins are useful for generating antibodies against phospholipase-like enzyme polypeptide amino acid sequences and for use in various assay systems. For example, fusion proteins can be used to identify proteins which interact with portions of a phospholipase-like enzyme polypeptide. Protein affinity chromatography or library-based assays for protein-protein interactions, such as the yeast two-hybrid or phage display systems, can be used for this purpose. Such methods are well known in the art and also can be used as drug screens.

A phospholipase-like enzyme polypeptide fusion protein comprises two polypeptide segments fused together by means of a peptide bond. The first polypeptide segment comprises at least 6, 10, 15, 20, 25, 50, 75, 100, 125, 150, 175, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000, 1050, 1100, 1150, 1200, or 1210 contiguous amino acids of SEQ ID NO:2 or of a biologically active variant, such as those described above. The first polypeptide segment also can comprise full-length phospholipase-like enzyme protein.

The second polypeptide segment can be a full-length protein or a protein fragment. Proteins commonly used in fusion protein construction include β -galactosidase, β -glucuronidase, green fluorescent protein (GFP), autofluorescent proteins, including blue fluorescent protein (BFP), glutathione-S-transferase (GST), luciferase, horseradish peroxidase (HRP), and chloramphenicol acetyltransferase (CAT). Additionally, epitope tags are used in fusion protein constructions, including histidine (His) tags, FLAG tags, influenza hemagglutinin (HA) tags, Myc tags, VSV-G tags, and thioredoxin (Trx) tags. Other fusion constructions can include maltose binding protein (MBP), S-tag, Lex a DNA binding domain (DBD) fusions, GAL4 DNA binding domain fusions, and herpes simplex virus (HSV) BP16 protein fusions. A fusion protein also can be engineered to contain a cleavage site located between the phospholipase-like enzyme polypeptide-encoding sequence and the heterologous protein sequence, so that the phospholipase-like enzyme polypeptide can be cleaved and purified away from the heterologous moiety.

5 A fusion protein can be synthesized chemically, as is known in the art. Preferably,
a fusion protein is produced by covalently linking two polypeptide segments or by standard
procedures in the art of molecular biology. Recombinant DNA methods can be used to
prepare fusion proteins, for example, by making a DNA construct which comprises coding
sequences selected from the complement of SEQ ID NO:1 in proper reading frame with
nucleotides encoding the second polypeptide segment and expressing the DNA construct
in a host cell, as is known in the art. Many kits for constructing fusion proteins are
available from companies such as Promega Corporation (Madison, WI), Stratagene (La
Jolla, CA), CLONTECH (Mountain View, CA), Santa Cruz Biotechnology (Santa Cruz,
10 CA), MBL International Corporation (MIC; Watertown, MA), and Quantum
Biotechnologies (Montreal, Canada; 1-888-DNA-KITS).

Identification of Species Homologs

15 Species homologs of human phospholipase-like enzyme polypeptide can be
obtained using phospholipase-like enzyme polypeptide polynucleotides (described below)
to make suitable probes or primers for screening cDNA expression libraries from other
species, such as mice, monkeys, or yeast, identifying cDNAs which encode homologs of
phospholipase-like enzyme polypeptide, and expressing the cDNAs as is known in the art.

Polynucleotides

20 A phospholipase-like enzyme polynucleotide can be single- or double-stranded and
comprises a coding sequence or the complement of a coding sequence for a phospholipase-
like enzyme polypeptide. A partial coding sequence for human phospholipase-like enzyme
is shown in SEQ ID NO:1.

25 Degenerate nucleotide sequences encoding human phospholipase-like enzyme
polypeptides, as well as homologous nucleotide sequences which are at least about 50, 55,
60, 65, 70, preferably about 75, 90, 96, or 98% identical to the nucleotide sequence shown
in SEQ ID NO:1 or its complement also are phospholipase-like enzyme polynucleotides.

Percent sequence identity between the sequences of two polynucleotides is determined using computer programs such as ALIGN which employ the FASTA algorithm, using an affine gap search with a gap open penalty of -12 and a gap extension penalty of -2. Complementary DNA (cDNA) molecules, species homologs, and variants of phospholipase-like enzyme polynucleotides which encode biologically active phospholipase-like enzyme polypeptides also are phospholipase-like enzyme polynucleotides.

Identification of Polynucleotide Variants and Homologs

Variants and homologs of the phospholipase-like enzyme polynucleotides described above also are phospholipase-like enzyme polynucleotides. Typically, homologous phospholipase-like enzyme polynucleotide sequences can be identified by hybridization of candidate polynucleotides to known phospholipase-like enzyme polynucleotides under stringent conditions, as is known in the art. For example, using the following wash conditions--2X SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0), 0.1% SDS, room temperature twice, 30 minutes each; then 2X SSC, 0.1% SDS, 50 °C once, 30 minutes; then 2X SSC, room temperature twice, 10 minutes each--homologous sequences can be identified which contain at most about 25-30% basepair mismatches. More preferably, homologous nucleic acid strands contain 15-25% basepair mismatches, even more preferably 5-15% basepair mismatches.

Species homologs of the phospholipase-like enzyme polynucleotides disclosed herein also can be identified by making suitable probes or primers and screening cDNA expression libraries from other species, such as mice, monkeys, or yeast. Human variants of phospholipase-like enzyme polynucleotides can be identified, for example, by screening human cDNA expression libraries. It is well known that the T_m of a double-stranded DNA decreases by 1-1.5 °C with every 1% decrease in homology (Bonner *et al.*, *J. Mol. Biol.* 81, 123 (1973). Variants of human phospholipase-like enzyme polynucleotides or phospholipase-like enzyme polynucleotides of other species can therefore be identified by hybridizing a putative homologous phospholipase-like enzyme polynucleotide with a

polynucleotide having a nucleotide sequence of SEQ ID NO:1 or the complement thereof to form a test hybrid. The melting temperature of the test hybrid is compared with the melting temperature of a hybrid comprising polynucleotides having perfectly complementary nucleotide sequences, and the number or percent of basepair mismatches within the test hybrid is calculated.

Nucleotide sequences which hybridize to phospholipase-like enzyme polynucleotides or their complements following stringent hybridization and/or wash conditions also are phospholipase-like enzyme polynucleotides. Stringent wash conditions are well known and understood in the art and are disclosed, for example, in Sambrook *et al.*, MOLECULAR CLONING: A LABORATORY MANUAL, 2d ed., 1989, at pages 9.50-9.51.

Typically, for stringent hybridization conditions a combination of temperature and salt concentration should be chosen that is approximately 12-20 °C below the calculated T_m of the hybrid under study. The T_m of a hybrid between a phospholipase-like enzyme polynucleotide having a nucleotide sequence shown in SEQ ID NO:1 or the complement thereof and a polynucleotide sequence which is at least about 50, preferably about 75, 90, 96, or 98% identical to one of those nucleotide sequences can be calculated, for example, using the equation of Bolton and McCarthy, *Proc. Natl. Acad. Sci. U.S.A.* 48, 1390 (1962):

$$T_m = 81.5\text{ }^{\circ}\text{C} - 16.6(\log_{10}[\text{Na}^+]) + 0.41(\%G + C) - 0.63(\%\text{formamide}) - 600/l,$$

where l = the length of the hybrid in basepairs.

Stringent wash conditions include, for example, 4X SSC at 65 °C, or 50% formamide, 4X SSC at 42 °C, or 0.5X SSC, 0.1% SDS at 65 °C. Highly stringent wash conditions include, for example, 0.2X SSC at 65 °C.

Preparation of Polynucleotides

A phospholipase-like enzyme polynucleotide can be isolated free of other cellular components such as membrane components, proteins, and lipids. Polynucleotides can be made by a cell and isolated using standard nucleic acid purification techniques, or synthesized using an amplification technique, such as the polymerase chain reaction (PCR),

or by using an automatic synthesizer. Methods for isolating polynucleotides are routine and are known in the art. Any such technique for obtaining a polynucleotide can be used to obtain isolated phospholipase-like enzyme polynucleotides. For example, restriction enzymes and probes can be used to isolate polynucleotide fragments which comprises phospholipase-like nucleotide sequences. Isolated polynucleotides are in preparations which are free or at least 70, 80, or 90% free of other molecules.

Human phospholipase-like enzyme cDNA molecules can be made with standard molecular biology techniques, using phospholipase-like enzyme mRNA as a template. Human phospholipase-like enzyme cDNA molecules can thereafter be replicated using molecular biology techniques known in the art and disclosed in manuals such as Sambrook *et al.* (1989). An amplification technique, such as PCR, can be used to obtain additional copies of polynucleotides of the invention, using either human genomic DNA or cDNA as a template.

Alternatively, synthetic chemistry techniques can be used to synthesize phospholipase-like enzyme polynucleotides. The degeneracy of the genetic code allows alternate nucleotide sequences to be synthesized which will encode a phospholipase-like enzyme polypeptide having, for example, an amino acid sequence shown in SEQ ID NO:2 or a biologically active variant thereof.

Extending Polynucleotides

The partial sequence disclosed herein can be used to identify the corresponding full length gene from which it was derived. The partial sequences can be nick-translated or end-labeled with ^{32}P using polynucleotide kinase using labeling methods known to those with skill in the art (BASIC METHODS IN MOLECULAR BIOLOGY, Davis *et al.*, eds., Elsevier Press, N.Y., 1986). A lambda library prepared from human tissue can be directly screened with the labeled sequences of interest or the library can be converted en masse to pBluescript (Stratagene Cloning Systems, La Jolla, Calif. 92037) to facilitate bacterial

colony screening (see Sambrook *et al.*, MOLECULAR CLONING: A LABORATORY MANUAL, Cold Spring Harbor Laboratory Press (1989, pg. 1.20).

Both methods are well known in the art. Briefly, filters with bacterial colonies containing the library in pBluescript or bacterial lawns containing lambda plaques are denatured, and the DNA is fixed to the filters. The filters are hybridized with the labeled probe using hybridization conditions described by Davis *et al.*, 1986. The partial sequences, cloned into lambda or pBluescript, can be used as positive controls to assess background binding and to adjust the hybridization and washing stringencies necessary for accurate clone identification. The resulting autoradiograms are compared to duplicate plates of colonies or plaques; each exposed spot corresponds to a positive colony or plaque. The colonies or plaques are selected, expanded and the DNA is isolated from the colonies for further analysis and sequencing.

Positive cDNA clones are analyzed to determine the amount of additional sequence they contain using PCR with one primer from the partial sequence and the other primer from the vector. Clones with a larger vector-insert PCR product than the original partial sequence are analyzed by restriction digestion and DNA sequencing to determine whether they contain an insert of the same size or similar as the mRNA size determined from Northern blot Analysis.

Once one or more overlapping cDNA clones are identified, the complete sequence of the clones can be determined, for example after exonuclease III digestion (McCombie *et al.*, *Methods* 3, 33-40, 1991). A series of deletion clones are generated, each of which is sequenced. The resulting overlapping sequences are assembled into a single contiguous sequence of high redundancy (usually three to five overlapping sequences at each nucleotide position), resulting in a highly accurate final sequence.

Various PCR-based methods can be used to extend the nucleic acid sequences disclosed herein to detect upstream sequences such as promoters and regulatory elements. For example, restriction-site PCR uses universal primers to retrieve unknown sequence adjacent to a known locus (Sarkar, *PCR Methods Applic.* 2, 318-322, 1993). Genomic

DNA is first amplified in the presence of a primer to a linker sequence and a primer specific to the known region. The amplified sequences are then subjected to a second round of PCR with the same linker primer and another specific primer internal to the first one. Products of each round of PCR are transcribed with an appropriate RNA polymerase and sequenced using reverse transcriptase.

Inverse PCR also can be used to amplify or extend sequences using divergent primers based on a known region (Triglia *et al.*, *Nucleic Acids Res.* 16, 8186, 1988). Primers can be designed using commercially available software, such as OLIGO 4.06 Primer Analysis software (National Biosciences Inc., Plymouth, Minn.), to be 22-30 nucleotides in length, to have a GC content of 50% or more, and to anneal to the target sequence at temperatures about 68-72 °C. The method uses several restriction enzymes to generate a suitable fragment in the known region of a gene. The fragment is then circularized by intramolecular ligation and used as a PCR template.

Another method which can be used is capture PCR, which involves PCR amplification of DNA fragments adjacent to a known sequence in human and yeast artificial chromosome DNA (Lagerstrom *et al.*, *PCR Methods Applic.* 1, 111-119, 1991). In this method, multiple restriction enzyme digestions and ligations also can be used to place an engineered double-stranded sequence into an unknown fragment of the DNA molecule before performing PCR.

Another method which can be used to retrieve unknown sequences is that of Parker *et al.*, *Nucleic Acids Res.* 19, 3055-3060, 1991). Additionally, PCR, nested primers, and PROMOTERFINDER libraries (CLONTECH, Palo Alto, Calif.) can be used to walk genomic DNA (CLONTECH, Palo Alto, Calif.). This process avoids the need to screen libraries and is useful in finding intron/exon junctions.

When screening for full-length cDNAs, it is preferable to use libraries that have been size-selected to include larger cDNAs. Randomly-primed libraries are preferable, in that they will contain more sequences which contain the 5' regions of genes. Use of a randomly primed library may be especially preferable for situations in which an oligo d(T)

library does not yield a full-length cDNA. Genomic libraries can be useful for extension of sequence into 5' non-transcribed regulatory regions.

Commercially available capillary electrophoresis systems can be used to analyze the size or confirm the nucleotide sequence of PCR or sequencing products. For example, capillary sequencing can employ flowable polymers for electrophoretic separation, four different fluorescent dyes (one for each nucleotide) which are laser activated, and detection of the emitted wavelengths by a charge coupled device camera. Output/light intensity can be converted to electrical signal using appropriate software (*e.g.* GENOTYPER and Sequence NAVIGATOR, Perkin Elmer), and the entire process from loading of samples to computer analysis and electronic data display can be computer controlled. Capillary electrophoresis is especially preferable for the sequencing of small pieces of DNA which might be present in limited amounts in a particular sample.

Obtaining Polypeptides

Human phospholipase-like enzyme polypeptides can be obtained, for example, by purification from human cells, by expression of phospholipase-like enzyme polynucleotides, or by direct chemical synthesis.

Protein Purification

Human phospholipase-like enzyme polypeptides can be purified from any cell which expresses the enzyme, including host cells which have been transfected with phospholipase-like enzyme expression constructs. A purified phospholipase-like enzyme polypeptide is separated from other compounds which normally associate with the phospholipase-like enzyme polypeptide in the cell, such as certain proteins, carbohydrates, or lipids, using methods well-known in the art. Such methods include, but are not limited to, size exclusion chromatography, ammonium sulfate fractionation, ion exchange chromatography, affinity chromatography, and preparative gel electrophoresis. A preparation of purified phospholipase-like enzyme polypeptides is at least 80% pure;

preferably, the preparations are 90%, 95%, or 99% pure. Purity of the preparations can be assessed by any means known in the art, such as SDS-polyacrylamide gel electrophoresis.

Expression of Polynucleotides

To express a phospholipase-like enzyme polynucleotide, the polynucleotide can be inserted into an expression vector which contains the necessary elements for the transcription and translation of the inserted coding sequence. Methods which are well known to those skilled in the art can be used to construct expression vectors containing sequences encoding phospholipase-like enzyme polypeptides and appropriate transcriptional and translational control elements. These methods include *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* genetic recombination. Such techniques are described, for example, in Sambrook *et al.* (1989) and in Ausubel *et al.*, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley & Sons, New York, N.Y., 1989.

A variety of expression vector/host systems can be utilized to contain and express sequences encoding a phospholipase-like enzyme polypeptide. These include, but are not limited to, microorganisms, such as bacteria transformed with recombinant bacteriophage, plasmid, or cosmid DNA expression vectors; yeast transformed with yeast expression vectors, insect cell systems infected with virus expression vectors (*e.g.*, baculovirus), plant cell systems transformed with virus expression vectors (*e.g.*, cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or with bacterial expression vectors (*e.g.*, Ti or pBR322 plasmids), or animal cell systems.

The control elements or regulatory sequences are those non-translated regions of the vector -- enhancers, promoters, 5' and 3' untranslated regions -- which interact with host cellular proteins to carry out transcription and translation. Such elements can vary in their strength and specificity. Depending on the vector system and host utilized, any number of suitable transcription and translation elements, including constitutive and inducible promoters, can be used. For example, when cloning in bacterial systems, inducible promoters such as the hybrid lacZ promoter of the BLUESCRIPT phagemid (Stratagene,

LaJolla, Calif.) or pSPORT1 plasmid (Life Technologies) and the like can be used. The baculovirus polyhedrin promoter can be used in insect cells. Promoters or enhancers derived from the genomes of plant cells (e.g., heat shock, RUBISCO, and storage protein genes) or from plant viruses (e.g., viral promoters or leader sequences) can be cloned into the vector. In mammalian cell systems, promoters from mammalian genes or from mammalian viruses are preferable. If it is necessary to generate a cell line that contains multiple copies of a nucleotide sequence encoding a phospholipase-like enzyme polypeptide, vectors based on SV40 or EBV can be used with an appropriate selectable marker.

Bacterial and Yeast Expression Systems

In bacterial systems, a number of expression vectors can be selected depending upon the use intended for the phospholipase-like enzyme polypeptide. For example, when a large quantity of a phospholipase-like enzyme polypeptide is needed for the induction of antibodies, vectors which direct high level expression of fusion proteins that are readily purified can be used. Such vectors include, but are not limited to, multifunctional *E. coli* cloning and expression vectors such as BLUESCRIPT (Stratagene). In a BLUESCRIPT vector, a sequence encoding the phospholipase-like enzyme polypeptide can be ligated into the vector in frame with sequences for the amino-terminal Met and the subsequent 7 residues of β -galactosidase so that a hybrid protein is produced. pIN vectors (Van Heeke & Schuster, *J. Biol. Chem.* 264, 5503-5509, 1989) or pGEX vectors (Promega, Madison, Wis.) also can be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. Proteins made in such systems can be designed to include heparin, thrombin, or factor Xa protease cleavage sites so that the cloned polypeptide of interest can be released from the GST moiety at will.

In the yeast *Saccharomyces cerevisiae*, a number of vectors containing constitutive or inducible promoters such as alpha factor, alcohol oxidase, and PGH can be used. For

reviews, see Ausubel *et al.* (1989) and Grant *et al.*, *Methods Enzymol.* 153, 516-544, 1987.

Plant and Insect Expression Systems

If plant expression vectors are used, the expression of sequences encoding phospholipase-like enzyme polypeptides can be driven by any of a number of promoters. For example, viral promoters such as the 35S and 19S promoters of CaMV can be used alone or in combination with the omega leader sequence from TMV (Takamatsu, *EMBO J.* 6, 307-311, 1987). Alternatively, plant promoters such as the small subunit of RUBISCO or heat shock promoters can be used (Coruzzi *et al.*, *EMBO J.* 3, 1671-1680, 1984; Broglie *et al.*, *Science* 224, 838-843, 1984; Winter *et al.*, *Results Probl. Cell Differ.* 17, 85-105, 1991). These constructs can be introduced into plant cells by direct DNA transformation or by pathogen-mediated transfection. Such techniques are described in a number of generally available reviews (*e.g.*, Hobbs or Murray, in MCGRAW HILL YEARBOOK OF SCIENCE AND TECHNOLOGY, McGraw Hill, New York, N.Y., pp. 191-196, 1992).

An insect system also can be used to express a phospholipase-like enzyme polypeptide. For example, in one such system *Autographa californica* nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes in *Spodoptera frugiperda* cells or in *Trichoplusia* larvae. Sequences encoding phospholipase-like enzyme polypeptides can be cloned into a non-essential region of the virus, such as the polyhedrin gene, and placed under control of the polyhedrin promoter. Successful insertion of phospholipase-like enzyme polypeptides will render the polyhedrin gene inactive and produce recombinant virus lacking coat protein. The recombinant viruses can then be used to infect *S. frugiperda* cells or *Trichoplusia* larvae in which phospholipase-like enzyme polypeptides can be expressed (Engelhard *et al.*, *Proc. Nat. Acad. Sci.* 91, 3224-3227, 1994).

Mammalian Expression Systems

A number of viral-based expression systems can be used to express phospholipase-like enzyme polypeptides in mammalian host cells. For example, if an adenovirus is used as an expression vector, sequences encoding phospholipase-like enzyme polypeptides can

be ligated into an adenovirus transcription/translation complex comprising the late promoter and tripartite leader sequence. Insertion in a non-essential E1 or E3 region of the viral genome can be used to obtain a viable virus which is capable of expressing a phospholipase-like enzyme polypeptide in infected host cells (Logan & Shenk, *Proc. Natl. Acad. Sci.* 81, 3655-3659, 1984). If desired, transcription enhancers, such as the Rous sarcoma virus (RSV) enhancer, can be used to increase expression in mammalian host cells.

Human artificial chromosomes (HACs) also can be used to deliver larger fragments of DNA than can be contained and expressed in a plasmid. HACs of 6M to 10M are constructed and delivered to cells via conventional delivery methods (e.g., liposomes, polycationic amino polymers, or vesicles).

Specific initiation signals also can be used to achieve more efficient translation of sequences encoding phospholipase-like enzyme polypeptides. Such signals include the ATG initiation codon and adjacent sequences. In cases where sequences encoding a phospholipase-like enzyme polypeptide, its initiation codon, and upstream sequences are inserted into the appropriate expression vector, no additional transcriptional or translational control signals may be needed. However, in cases where only coding sequence, or a fragment thereof, is inserted, exogenous translational control signals (including the ATG initiation codon) should be provided. The initiation codon should be in the correct reading frame to ensure translation of the entire insert. Exogenous translational elements and initiation codons can be of various origins, both natural and synthetic. The efficiency of expression can be enhanced by the inclusion of enhancers which are appropriate for the particular cell system which is used (see Scharf *et al.*, *Results Probl. Cell Differ.* 20, 125-162, 1994).

Host Cells

A host cell strain can be chosen for its ability to modulate the expression of the inserted sequences or to process the expressed phospholipase-like enzyme polypeptide in the desired fashion. Such modifications of the polypeptide include, but are not limited to, acetylation, carboxylation, glycosylation, phosphorylation, lipidation, and acylation.

Post-translational processing which cleaves a "prepro" form of the polypeptide also can be used to facilitate correct insertion, folding and/or function. Different host cells which have specific cellular machinery and characteristic mechanisms for post-translational activities (e.g., CHO, HeLa, MDCK, HEK293, and WI38), are available from the American Type Culture Collection (ATCC; 10801 University Boulevard, Manassas, VA 20110-2209) and can be chosen to ensure the correct modification and processing of the foreign protein.

Stable expression is preferred for long-term, high-yield production of recombinant proteins. For example, cell lines which stably express phospholipase-like enzyme polypeptides can be transformed using expression vectors which can contain viral origins of replication and/or endogenous expression elements and a selectable marker gene on the same or on a separate vector. Following the introduction of the vector, cells can be allowed to grow for 1-2 days in an enriched medium before they are switched to a selective medium. The purpose of the selectable marker is to confer resistance to selection, and its presence allows growth and recovery of cells which successfully express the introduced phospholipase-like enzyme sequences. Resistant clones of stably transformed cells can be proliferated using tissue culture techniques appropriate to the cell type. See, for example, ANIMAL CELL CULTURE, R.I. Freshney, ed., 1986.

Any number of selection systems can be used to recover transformed cell lines.

These include, but are not limited to, the herpes simplex virus thymidine kinase (Wigler *et al.*, *Cell* 11, 223-32, 1977) and adenine phosphoribosyltransferase (Lowy *et al.*, *Cell* 22, 817-23, 1980) genes which can be employed in *tk* or *aprt* cells, respectively. Also, antimetabolite, antibiotic, or herbicide resistance can be used as the basis for selection. For example, *dhfr* confers resistance to methotrexate (Wigler *et al.*, *Proc. Natl. Acad. Sci.* 77, 3567-70, 1980), *npt* confers resistance to the aminoglycosides, neomycin and G-418 (Colbere-Garapin *et al.*, *J. Mol. Biol.* 150, 1-14, 1981), and *als* and *pat* confer resistance to chlorsulfuron and phosphinotricin acetyltransferase, respectively (Murray, 1992, *supra*). Additional selectable genes have been described. For example, *trpB* allows cells to utilize indole in place of tryptophan, or *hisD*, which allows cells to utilize histinol in place of

histidine (Hartman & Mulligan, *Proc. Natl. Acad. Sci.* 85, 8047-51, 1988). Visible markers such as anthocyanins, β -glucuronidase and its substrate GUS, and luciferase and its substrate luciferin, can be used to identify transformants and to quantify the amount of transient or stable protein expression attributable to a specific vector system (Rhodes *et al.*, *Methods Mol. Biol.* 55, 121-131, 1995).

Detecting Expression

Although the presence of marker gene expression suggests that the phospholipase-like enzyme polynucleotide is also present, its presence and expression may need to be confirmed. For example, if a sequence encoding a phospholipase-like enzyme polypeptide is inserted within a marker gene sequence, transformed cells containing sequences which encode a phospholipase-like enzyme polypeptide can be identified by the absence of marker gene function. Alternatively, a marker gene can be placed in tandem with a sequence encoding a phospholipase-like enzyme polypeptide under the control of a single promoter. Expression of the marker gene in response to induction or selection usually indicates expression of the phospholipase-like enzyme polynucleotide.

Alternatively, host cells which contain a phospholipase-like enzyme polynucleotide and which express a phospholipase-like enzyme polypeptide can be identified by a variety of procedures known to those of skill in the art. These procedures include, but are not limited to, DNA-DNA or DNA-RNA hybridizations and protein bioassay or immunoassay techniques which include membrane, solution, or chip-based technologies for the detection and/or quantification of nucleic acid or protein. For example, the presence of a polynucleotide sequence encoding a phospholipase-like enzyme polypeptide can be detected by DNA-DNA or DNA-RNA hybridization or amplification using probes or fragments or fragments of polynucleotides encoding a phospholipase-like enzyme polypeptide. Nucleic acid amplification-based assays involve the use of oligonucleotides selected from sequences encoding a phospholipase-like enzyme polypeptide to detect transformants which contain a phospholipase-like enzyme polynucleotide.

A variety of protocols for detecting and measuring the expression of a

phospholipase-like enzyme polypeptide, using either polyclonal or monoclonal antibodies specific for the polypeptide, are known in the art. Examples include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), and fluorescence activated cell sorting (FACS). A two-site, monoclonal-based immunoassay using monoclonal antibodies reactive to two non-interfering epitopes on a phospholipase-like enzyme polypeptide can be used, or a competitive binding assay can be employed. These and other assays are described in Hampton *et al.*, SEROLOGICAL METHODS: A LABORATORY MANUAL, APS Press, St. Paul, Minn., 1990) and Maddox *et al.*, *J. Exp. Med.* 158, 1211-1216, 1983).

A wide variety of labels and conjugation techniques are known by those skilled in the art and can be used in various nucleic acid and amino acid assays. Means for producing labeled hybridization or PCR probes for detecting sequences related to polynucleotides encoding phospholipase-like enzyme polypeptides include oligolabeling, nick translation, end-labeling, or PCR amplification using a labeled nucleotide. Alternatively, sequences encoding a phospholipase-like enzyme polypeptide can be cloned into a vector for the production of an mRNA probe. Such vectors are known in the art, are commercially available, and can be used to synthesize RNA probes *in vitro* by addition of labeled nucleotides and an appropriate RNA polymerase such as T7, T3, or SP6. These procedures can be conducted using a variety of commercially available kits (Amersham Pharmacia Biotech, Promega, and US Biochemical). Suitable reporter molecules or labels which can be used for ease of detection include radionuclides, enzymes, and fluorescent, chemiluminescent, or chromogenic agents, as well as substrates, cofactors, inhibitors, magnetic particles, and the like.

Expression and Purification of Polypeptides

Host cells transformed with nucleotide sequences encoding a phospholipase-like enzyme polypeptide can be cultured under conditions suitable for the expression and recovery of the protein from cell culture. The polypeptide produced by a transformed cell can be secreted or contained intracellularly depending on the sequence and/or the vector used. As will be understood by those of skill in the art, expression vectors containing

polynucleotides which encode phospholipase-like enzyme polypeptides can be designed to contain signal sequences which direct secretion of soluble phospholipase-like enzyme polypeptides through a prokaryotic or eukaryotic cell membrane or which direct the membrane insertion of membrane-bound phospholipase-like enzyme polypeptide.

5 As discussed above, other constructions can be used to join a sequence encoding a phospholipase-like enzyme polypeptide to a nucleotide sequence encoding a polypeptide domain which will facilitate purification of soluble proteins. Such purification facilitating domains include, but are not limited to, metal chelating peptides such as histidine-tryptophan modules that allow purification on immobilized metals, protein A
10 domains that allow purification on immobilized immunoglobulin, and the domain utilized in the FLAGS extension/affinity purification system (Immunex Corp., Seattle, Wash.). Inclusion of cleavable linker sequences such as those specific for Factor Xa or enterokinase (Invitrogen, San Diego, CA) between the purification domain and the phospholipase-like enzyme polypeptide also can be used to facilitate purification. One such expression vector
15 provides for expression of a fusion protein containing a phospholipase-like enzyme polypeptide and 6 histidine residues preceding a thioredoxin or an enterokinase cleavage site. The histidine residues facilitate purification by IMAC (immobilized metal ion affinity chromatography, as described in Porath *et al.*, *Prot. Exp. Purif.* 3, 263-281, 1992), while the enterokinase cleavage site provides a means for purifying the phospholipase-like
20 enzyme polypeptide from the fusion protein. Vectors which contain fusion proteins are disclosed in Kroll *et al.*, *DNA Cell Biol.* 12, 441-453, 1993.

Chemical Synthesis

Sequences encoding a phospholipase-like enzyme polypeptide can be synthesized, in whole or in part, using chemical methods well known in the art (see Caruthers *et al.*,
25 *Nucl. Acids Res. Symp. Ser.* 215-223, 1980; Horn *et al.* *Nucl. Acids Res. Symp. Ser.* 225-232, 1980). Alternatively, a phospholipase-like enzyme polypeptide itself can be produced using chemical methods to synthesize its amino acid sequence, such as by direct peptide synthesis using solid-phase techniques (Merrifield, *J. Am. Chem. Soc.* 85,

2149-2154, 1963; Roberge *et al.*, *Science* 269, 202-204, 1995). Protein synthesis can be performed using manual techniques or by automation. Automated synthesis can be achieved, for example, using Applied Biosystems 431A Peptide Synthesizer (Perkin Elmer). Optionally, fragments of phospholipase-like enzyme polypeptides can be separately synthesized and combined using chemical methods to produce a full-length molecule.

The newly synthesized peptide can be substantially purified by preparative high performance liquid chromatography (*e.g.*, Creighton, *PROTEINS: STRUCTURES AND MOLECULAR PRINCIPLES*, WH Freeman and Co., New York, N.Y., 1983). The composition of a synthetic phospholipase-like enzyme polypeptide can be confirmed by amino acid analysis or sequencing (*e.g.*, the Edman degradation procedure; *see* Creighton, *supra*). Additionally, any portion of the amino acid sequence of the phospholipase-like enzyme polypeptide can be altered during direct synthesis and/or combined using chemical methods with sequences from other proteins to produce a variant polypeptide or a fusion protein.

Production of Altered Polypeptides

As will be understood by those of skill in the art, it may be advantageous to produce phospholipase-like enzyme polypeptide-encoding nucleotide sequences possessing non-naturally occurring codons. For example, codons preferred by a particular prokaryotic or eukaryotic host can be selected to increase the rate of protein expression or to produce an RNA transcript having desirable properties, such as a half-life which is longer than that of a transcript generated from the naturally occurring sequence.

The nucleotide sequences disclosed herein can be engineered using methods generally known in the art to alter phospholipase-like enzyme polypeptide-encoding sequences for a variety of reasons, including but not limited to, alterations which modify the cloning, processing, and/or expression of the polypeptide or mRNA product. DNA shuffling by random fragmentation and PCR reassembly of gene fragments and synthetic oligonucleotides can be used to engineer the nucleotide sequences. For example, site-directed mutagenesis can be used to insert new restriction sites, alter glycosylation

patterns, change codon preference, produce splice variants, introduce mutations, and so forth.

Antibodies

5 Any type of antibody known in the art can be generated to bind specifically to an epitope of a phospholipase-like enzyme polypeptide. "Antibody" as used herein includes intact immunoglobulin molecules, as well as fragments thereof, such as Fab, F(ab')₂, and Fv, which are capable of binding an epitope of a phospholipase-like enzyme polypeptide. Typically, at least 6, 8, 10, or 12 contiguous amino acids are required to form an epitope. However, epitopes which involve non-contiguous amino acids may require more, *e.g.*, at 10 least 15, 25, or 50 amino acids.

An antibody which specifically binds to an epitope of a phospholipase-like enzyme polypeptide can be used therapeutically, as well as in immunochemical assays, such as Western blots, ELISAs, radioimmunoassays, immunohistochemical assays, 15 immunoprecipitations, or other immunochemical assays known in the art. Various immunoassays can be used to identify antibodies having the desired specificity. Numerous protocols for competitive binding or immunoradiometric assays are well known in the art. Such immunoassays typically involve the measurement of complex formation between an immunogen and an antibody which specifically binds to the immunogen.

20 Typically, an antibody which specifically binds to a phospholipase-like enzyme polypeptide provides a detection signal at least 5-, 10-, or 20-fold higher than a detection signal provided with other proteins when used in an immunochemical assay. Preferably, antibodies which specifically bind to phospholipase-like polypeptides do not detect other proteins in immunochemical assays and can immunoprecipitate a phospholipase-like 25 enzyme polypeptide from solution.

Human phospholipase-like enzyme polypeptides can be used to immunize a mammal, such as a mouse, rat, rabbit, guinea pig, monkey, or human, to produce polyclonal

antibodies. If desired, a phospholipase-like enzyme polypeptide can be conjugated to a carrier protein, such as bovine serum albumin, thyroglobulin, and keyhole limpet hemocyanin. Depending on the host species, various adjuvants can be used to increase the immunological response. Such adjuvants include, but are not limited to, Freund's adjuvant, mineral gels (*e.g.*, aluminum hydroxide), and surface active substances (*e.g.* lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, and dinitrophenol). Among adjuvants used in humans, BCG (*bacilli Calmette-Guerin*) and *Corynebacterium parvum* are especially useful.

Monoclonal antibodies which specifically bind to a phospholipase-like enzyme polypeptide can be prepared using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These techniques include, but are not limited to, the hybridoma technique, the human B-cell hybridoma technique, and the EBV-hybridoma technique (Kohler *et al.*, *Nature* 256, 495-497, 1985; Kozbor *et al.*, *J. Immunol. Methods* 81, 31-42, 1985; Cote *et al.*, *Proc. Natl. Acad. Sci.* 80, 2026-2030, 1983; Cole *et al.*, *Mol. Cell Biol.* 62, 109-120, 1984).

In addition, techniques developed for the production of "chimeric antibodies," the splicing of mouse antibody genes to human antibody genes to obtain a molecule with appropriate antigen specificity and biological activity, can be used (Morrison *et al.*, *Proc. Natl. Acad. Sci.* 81, 6851-6855, 1984; Neuberger *et al.*, *Nature* 312, 604-608, 1984; Takeda *et al.*, *Nature* 314, 452-454, 1985). Monoclonal and other antibodies also can be "humanized" to prevent a patient from mounting an immune response against the antibody when it is used therapeutically. Such antibodies may be sufficiently similar in sequence to human antibodies to be used directly in therapy or may require alteration of a few key residues. Sequence differences between rodent antibodies and human sequences can be minimized by replacing residues which differ from those in the human sequences by site directed mutagenesis of individual residues or by grating of entire complementarity determining regions. Alternatively, humanized antibodies can be produced using recombinant methods, as described in GB2188638B. Antibodies which specifically bind

to a phospholipase-like enzyme polypeptide can contain antigen binding sites which are either partially or fully humanized, as disclosed in U.S. 5,565,332.

Alternatively, techniques described for the production of single chain antibodies can be adapted using methods known in the art to produce single chain antibodies which specifically bind to phospholipase-like enzyme polypeptides. Antibodies with related specificity, but of distinct idiotypic composition, can be generated by chain shuffling from random combinatorial immunoglobulin libraries (Burton, *Proc. Natl. Acad. Sci.* 88, 11120-23, 1991).

Single-chain antibodies also can be constructed using a DNA amplification method, such as PCR, using hybridoma cDNA as a template (Thirion *et al.*, 1996, *Eur. J. Cancer Prev.* 5, 507-11). Single-chain antibodies can be mono- or bispecific, and can be bivalent or tetravalent. Construction of tetravalent, bispecific single-chain antibodies is taught, for example, in Coloma & Morrison, 1997, *Nat. Biotechnol.* 15, 159-63. Construction of bivalent, bispecific single-chain antibodies is taught in Mallender & Voss, 1994, *J. Biol. Chem.* 269, 199-206.

A nucleotide sequence encoding a single-chain antibody can be constructed using manual or automated nucleotide synthesis, cloned into an expression construct using standard recombinant DNA methods, and introduced into a cell to express the coding sequence, as described below. Alternatively, single-chain antibodies can be produced directly using, for example, filamentous phage technology (Verhaar *et al.*, 1995, *Int. J. Cancer* 61, 497-501; Nicholls *et al.*, 1993, *J. Immunol. Meth.* 165, 81-91).

Antibodies which specifically bind to phospholipase-like enzyme polypeptides also can be produced by inducing *in vivo* production in the lymphocyte population or by screening immunoglobulin libraries or panels of highly specific binding reagents as disclosed in the literature (Orlandi *et al.*, *Proc. Natl. Acad. Sci.* 86, 3833-3837, 1989; Winter *et al.*, *Nature* 349, 293-299, 1991).

Other types of antibodies can be constructed and used therapeutically in methods

of the invention. For example, chimeric antibodies can be constructed as disclosed in WO 93/03151. Binding proteins which are derived from immunoglobulins and which are multivalent and multispecific, such as the "diabodies" described in WO 94/13804, also can be prepared.

Antibodies according to the invention can be purified by methods well known in the art. For example, antibodies can be affinity purified by passage over a column to which a phospholipase-like enzyme polypeptide is bound. The bound antibodies can then be eluted from the column using a buffer with a high salt concentration.

Antisense Oligonucleotides

Antisense oligonucleotides are nucleotide sequences which are complementary to a specific DNA or RNA sequence. Once introduced into a cell, the complementary nucleotides combine with natural sequences produced by the cell to form complexes and block either transcription or translation. Preferably, an antisense oligonucleotide is at least 11 nucleotides in length, but can be at least 12, 15, 20, 25, 30, 35, 40, 45, or 50 or more nucleotides long. Longer sequences also can be used. Antisense oligonucleotide molecules can be provided in a DNA construct and introduced into a cell as described above to decrease the level of phospholipase-like enzyme gene products in the cell.

Antisense oligonucleotides can be deoxyribonucleotides, ribonucleotides, or a combination of both. Oligonucleotides can be synthesized manually or by an automated synthesizer, by covalently linking the 5' end of one nucleotide with the 3' end of another nucleotide with non-phosphodiester internucleotide linkages such as alkylphosphonates, phosphorothioates, phosphorodithioates, alkylphosphonothioates, alkylphosphonates, phosphoramidates, phosphate esters, carbamates, acetamidate, carboxymethyl esters, carbonates, and phosphate triesters. See Brown, *Meth. Mol. Biol.* 20, 1-8, 1994; Sonveaux, *Meth. Mol. Biol.* 26, 1-72, 1994; Uhlmann *et al.*, *Chem. Rev.* 90, 543-583, 1990.

Modifications of phospholipase-like enzyme gene expression can be obtained by

designing antisense oligonucleotides which will form duplexes to the control, 5', or regulatory regions of the phospholipase-like enzyme gene. Oligonucleotides derived from the transcription initiation site, *e.g.*, between positions -10 and +10 from the start site, are preferred. Similarly, inhibition can be achieved using "triple helix" base-pairing methodology. Triple helix pairing is useful because it causes inhibition of the ability of the double helix to open sufficiently for the binding of polymerases, transcription factors, or chaperons. Therapeutic advances using triplex DNA have been described in the literature (*e.g.*, Gee *et al.*, in Huber & Carr, MOLECULAR AND IMMUNOLOGIC APPROACHES, Futura Publishing Co., Mt. Kisco, N.Y., 1994). An antisense oligonucleotide also can be designed to block translation of mRNA by preventing the transcript from binding to ribosomes.

Precise complementarity is not required for successful complex formation between an antisense oligonucleotide and the complementary sequence of a phospholipase-like enzyme polynucleotide. Antisense oligonucleotides which comprise, for example, 2, 3, 4, or 5 or more stretches of contiguous nucleotides which are precisely complementary to a phospholipase-like enzyme polynucleotide, each separated by a stretch of contiguous nucleotides which are not complementary to adjacent phospholipase-like enzyme nucleotides, can provide sufficient targeting specificity for phospholipase-like enzyme mRNA. Preferably, each stretch of complementary contiguous nucleotides is at least 4, 5, 6, 7, or 8 or more nucleotides in length. Non-complementary intervening sequences are preferably 1, 2, 3, or 4 nucleotides in length. One skilled in the art can easily use the calculated melting point of an antisense-sense pair to determine the degree of mismatching which will be tolerated between a particular antisense oligonucleotide and a particular phospholipase-like enzyme polynucleotide sequence.

Antisense oligonucleotides can be modified without affecting their ability to hybridize to a phospholipase-like enzyme polynucleotide. These modifications can be internal or at one or both ends of the antisense molecule. For example, internucleoside phosphate linkages can be modified by adding cholesteryl or diamine moieties with varying numbers of carbon residues between the amino groups and terminal ribose. Modified bases

and/or sugars, such as arabinose instead of ribose, or a 3', 5'-substituted oligonucleotide in which the 3' hydroxyl group or the 5' phosphate group are substituted, also can be employed in a modified antisense oligonucleotide. These modified oligonucleotides can be prepared by methods well known in the art. See, e.g., Agrawal *et al.*, *Trends Biotechnol.* 10, 152-158, 1992; Uhlmann *et al.*, *Chem. Rev.* 90, 543-584, 1990; Uhlmann *et al.*, *Tetrahedron. Lett.* 215, 3539-3542, 1987.

Ribozymes

Ribozymes are RNA molecules with catalytic activity. See, e.g., Cech, *Science* 236, 1532-1539; 1987; Cech, *Ann. Rev. Biochem.* 59, 543-568; 1990, Cech, *Curr. Opin. Struct. Biol.* 2, 605-609; 1992, Couture & Stinchcomb, *Trends Genet.* 12, 510-515, 1996. Ribozymes can be used to inhibit gene function by cleaving an RNA sequence, as is known in the art (e.g., Haseloff *et al.*, U.S. Patent 5,641,673). The mechanism of ribozyme action involves sequence-specific hybridization of the ribozyme molecule to complementary target RNA, followed by endonucleolytic cleavage. Examples include engineered hammerhead motif ribozyme molecules that can specifically and efficiently catalyze endonucleolytic cleavage of specific nucleotide sequences.

The coding sequence of a phospholipase-like enzyme polynucleotide can be used to generate ribozymes which will specifically bind to mRNA transcribed from the phospholipase-like enzyme polynucleotide. Methods of designing and constructing ribozymes which can cleave other RNA molecules in trans in a highly sequence specific manner have been developed and described in the art (see Haseloff *et al.* *Nature* 334, 585-591, 1988). For example, the cleavage activity of ribozymes can be targeted to specific RNAs by engineering a discrete "hybridization" region into the ribozyme. The hybridization region contains a sequence complementary to the target RNA and thus specifically hybridizes with the target (see, for example, Gerlach *et al.*, EP 321,201).

Specific ribozyme cleavage sites within a phospholipase-like enzyme RNA target

can be identified by scanning the target molecule for ribozyme cleavage sites which include the following sequences: GUA, GUU, and GUC. Once identified, short RNA sequences of between 15 and 20 ribonucleotides corresponding to the region of the target RNA containing the cleavage site can be evaluated for secondary structural features which may render the target inoperable. Suitability of candidate phospholipase-like enzyme RNA targets also can be evaluated by testing accessibility to hybridization with complementary oligonucleotides using ribonuclease protection assays. Longer complementary sequences can be used to increase the affinity of the hybridization sequence for the target. The hybridizing and cleavage regions of the ribozyme can be integrally related such that upon hybridizing to the target RNA through the complementary regions, the catalytic region of the ribozyme can cleave the target.

Ribozymes can be introduced into cells as part of a DNA construct. Mechanical methods, such as microinjection, liposome-mediated transfection, electroporation, or calcium phosphate precipitation, can be used to introduce a ribozyme-containing DNA construct into cells in which it is desired to decrease phospholipase-like enzyme expression. Alternatively, if it is desired that the cells stably retain the DNA construct, the construct can be supplied on a plasmid and maintained as a separate element or integrated into the genome of the cells, as is known in the art. A ribozyme-encoding DNA construct can include transcriptional regulatory elements, such as a promoter element, an enhancer or UAS element, and a transcriptional terminator signal, for controlling transcription of ribozymes in the cells.

As taught in Haseloff *et al.*, U.S. Patent 5,641,673, ribozymes can be engineered so that ribozyme expression will occur in response to factors which induce expression of a target gene. Ribozymes also can be engineered to provide an additional level of regulation, so that destruction of mRNA occurs only when both a ribozyme and a target gene are induced in the cells.

Differentially Expressed Genes

Described herein are methods for the identification of genes whose products interact with human phospholipase-like enzyme. Such genes may represent genes which are differentially expressed in disorders including, but not limited to, cancer, inflammation, CNS disorders, cardiovascular disorders, chronic obstructive pulmonary disease, and obesity. Further, such genes may represent genes which are differentially regulated in response to manipulations relevant to the progression or treatment of such diseases. Additionally, such genes may have a temporally modulated expression, increased or decreased at different stages of tissue or organism development. A differentially expressed gene may also have its expression modulated under control versus experimental conditions. In addition, the human phospholipase-like enzyme gene or gene product may itself be tested for differential expression.

The degree to which expression differs in a normal versus a diseased state need only be large enough to be visualized via standard characterization techniques such as differential display techniques. Other such standard characterization techniques by which expression differences may be visualized include but are not limited to, quantitative RT (reverse transcriptase), PCR, and Northern analysis.

Identification of Differentially Expressed Genes

To identify differentially expressed genes total RNA or, preferably, mRNA is isolated from tissues of interest. For example, RNA samples are obtained from tissues of experimental subjects and from corresponding tissues of control subjects. Any RNA isolation technique which does not select against the isolation of mRNA may be utilized for the purification of such RNA samples. See, for example, Ausubel *et al.*, ed., CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley & Sons, Inc. New York, 1987-1993. Large numbers of tissue samples may readily be processed using techniques well known to those of skill in the art, such as, for example, the single-step RNA isolation process of Chomczynski, U.S. Patent 4,843,155.

Transcripts within the collected RNA samples which represent RNA produced by differentially expressed genes are identified by methods well known to those of skill in the

art. They include, for example, differential screening (Tedder *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 85, 208-12, 1988), subtractive hybridization (Hedrick *et al.*, *Nature* 308, 149-53; Lee *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 88, 2825, 1984), and, preferably, differential display (Liang & Pardee, *Science* 257, 967-71, 1992; U.S. Patent 5,262,311).

5 The differential expression information may itself suggest relevant methods for the treatment of disorders involving the human phospholipase-like enzyme. For example, treatment may include a modulation of expression of the differentially expressed genes and/or the gene encoding the human phospholipase-like enzyme. The differential expression information may indicate whether the expression or activity of the differentially
10 expressed gene or gene product or the human phospholipase-like enzyme gene or gene product are up-regulated or down-regulated.

Screening Methods

15 The invention provides assays for screening test compounds which bind to or modulate the activity of a phospholipase-like enzyme polypeptide or a phospholipase-like enzyme polynucleotide. A test compound preferably binds to a phospholipase-like enzyme polypeptide or polynucleotide. More preferably, a test compound decreases or increases phospholipase-like by at least about 10, preferably about 50, more preferably about 75, 90, or 100% relative to the absence of the test compound.

Test Compounds

20 Test compounds can be pharmacologic agents already known in the art or can be compounds previously unknown to have any pharmacological activity. The compounds can be naturally occurring or designed in the laboratory. They can be isolated from microorganisms, animals, or plants, and can be produced recombinantly, or synthesized by
25 chemical methods known in the art. If desired, test compounds can be obtained using any of the numerous combinatorial library methods known in the art, including but not limited to, biological libraries, spatially addressable parallel solid phase or solution phase libraries,

synthetic library methods requiring deconvolution, the “one-bead one-compound” library method, and synthetic library methods using affinity chromatography selection. The biological library approach is limited to polypeptide libraries, while the other four approaches are applicable to polypeptide, non-peptide oligomer, or small molecule libraries of compounds. See Lam, *Anticancer Drug Des.* 12, 145, 1997.

Methods for the synthesis of molecular libraries are well known in the art (see, for example, DeWitt *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 90, 6909, 1993; Erb *et al.* *Proc. Natl. Acad. Sci. U.S.A.* 91, 11422, 1994; Zuckermann *et al.*, *J. Med. Chem.* 37, 2678, 1994; Cho *et al.*, *Science* 261, 1303, 1993; Carell *et al.*, *Angew. Chem. Int. Ed. Engl.* 33, 2059, 1994; Carell *et al.*, *Angew. Chem. Int. Ed. Engl.* 33, 2061; Gallop *et al.*, *J. Med. Chem.* 37, 1233, 1994). Libraries of compounds can be presented in solution (see, e.g., Houghten, *BioTechniques* 13, 412-421, 1992), or on beads (Lam, *Nature* 354, 82-84, 1991), chips (Fodor, *Nature* 364, 555-556, 1993), bacteria or spores (Ladner, U.S. Patent 5,223,409), plasmids (Cull *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 89, 1865-1869, 1992), or phage (Scott & Smith, *Science* 249, 386-390, 1990; Devlin, *Science* 249, 404-406, 1990); Cwirla *et al.*, *Proc. Natl. Acad. Sci.* 97, 6378-6382, 1990; Felici, *J. Mol. Biol.* 222, 301-310, 1991; and Ladner, U.S. Patent 5,223,409).

High Throughput Screening

Test compounds can be screened for the ability to bind to phospholipase-like enzyme polypeptides or polynucleotides or to affect phospholipase-like enzyme activity or phospholipase-like enzyme gene expression using high throughput screening. Using high throughput screening, many discrete compounds can be tested in parallel so that large numbers of test compounds can be quickly screened. The most widely established techniques utilize 96-well microtiter plates. The wells of the microtiter plates typically require assay volumes that range from 50 to 500 μ l. In addition to the plates, many instruments, materials, pipettors, robotics, plate washers, and plate readers are commercially available to fit the 96-well format.

Alternatively, “free format assays,” or assays that have no physical barrier between

samples, can be used. For example, an assay using pigment cells (melanocytes) in a simple homogeneous assay for combinatorial peptide libraries is described by Jayawickreme *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 19, 1614-18 (1994). The cells are placed under agarose in petri dishes, then beads that carry combinatorial compounds are placed on the surface of the agarose. The combinatorial compounds are partially released the compounds from the beads. Active compounds can be visualized as dark pigment areas because, as the compounds diffuse locally into the gel matrix, the active compounds cause the cells to change colors.

Another example of a free format assay is described by Chelsky, "Strategies for Screening Combinatorial Libraries: Novel and Traditional Approaches," reported at the First Annual Conference of The Society for Biomolecular Screening in Philadelphia, Pa. (Nov. 7-10, 1995). Chelsky placed a simple homogenous enzyme assay for carbonic anhydrase inside an agarose gel such that the enzyme in the gel would cause a color change throughout the gel. Thereafter, beads carrying combinatorial compounds via a photolinker were placed inside the gel and the compounds were partially released by UV-light. Compounds that inhibited the enzyme were observed as local zones of inhibition having less color change.

Yet another example is described by Salmon *et al.*, *Molecular Diversity* 2, 57-63 (1996). In this example, combinatorial libraries were screened for compounds that had cytotoxic effects on cancer cells growing in agar.

Another high throughput screening method is described in Beutel *et al.*, U.S. Patent 5,976,813. In this method, test samples are placed in a porous matrix. One or more assay components are then placed within, on top of, or at the bottom of a matrix such as a gel, a plastic sheet, a filter, or other form of easily manipulated solid support. When samples are introduced to the porous matrix they diffuse sufficiently slowly, such that the assays can be performed without the test samples running together.

Binding Assays

For binding assays, the test compound is preferably a small molecule which binds to and occupies, for example, the active site of the phospholipase-like enzyme polypeptide, such that normal biological activity is prevented. Examples of such small molecules include, but are not limited to, small peptides or peptide-like molecules.

5 In binding assays, either the test compound or the phospholipase-like enzyme polypeptide can comprise a detectable label, such as a fluorescent, radioisotopic, chemiluminescent, or enzymatic label, such as horseradish peroxidase, alkaline phosphatase, or luciferase. Detection of a test compound which is bound to the phospholipase-like enzyme polypeptide can then be accomplished, for example, by direct
10 counting of radioemmission, by scintillation counting, or by determining conversion of an appropriate substrate to a detectable product.

Alternatively, binding of a test compound to a phospholipase-like enzyme polypeptide can be determined without labeling either of the interactants. For example, a microphysiometer can be used to detect binding of a test compound with a phospholipase-like enzyme polypeptide. A microphysiometer (*e.g.*, Cytosensor™) is an analytical
15 instrument that measures the rate at which a cell acidifies its environment using a light-addressable potentiometric sensor (LAPS). Changes in this acidification rate can be used as an indicator of the interaction between a test compound and a phospholipase-like enzyme polypeptide (McConnell *et al.*, *Science* 257, 1906-1912, 1992).

20 Determining the ability of a test compound to bind to a phospholipase-like enzyme polypeptide also can be accomplished using a technology such as real-time Bimolecular Interaction Analysis (BIA) (Sjolander & Urbaniczky, *Anal. Chem.* 63, 2338-2345, 1991, and Szabo *et al.*, *Curr. Opin. Struct. Biol.* 5, 699-705, 1995). BIA is a technology for studying biospecific interactions in real time, without labeling any of the interactants (*e.g.*,
25 BIAcore™). Changes in the optical phenomenon surface plasmon resonance (SPR) can be used as an indication of real-time reactions between biological molecules.

In yet another aspect of the invention, a phospholipase-like enzyme polypeptide can be used as a "bait protein" in a two-hybrid assay or three-hybrid assay (see, *e.g.*, U.S. Patent

5,283,317; Zervos *et al.*, *Cell* 72, 223-232, 1993; Madura *et al.*, *J. Biol. Chem.* 268, 12046-12054, 1993; Bartel *et al.*, *BioTechniques* 14, 920-924, 1993; Iwabuchi *et al.*, *Oncogene* 8, 1693-1696, 1993; and Brent W094/10300), to identify other proteins which bind to or interact with the phospholipase-like enzyme polypeptide and modulate its activity.

The two-hybrid system is based on the modular nature of most transcription factors, which consist of separable DNA-binding and activation domains. Briefly, the assay utilizes two different DNA constructs. For example, in one construct, polynucleotide encoding a phospholipase-like enzyme polypeptide can be fused to a polynucleotide encoding the DNA binding domain of a known transcription factor (*e.g.*, GAL-4). In the other construct a DNA sequence that encodes an unidentified protein ("prey" or "sample") can be fused to a polynucleotide that codes for the activation domain of the known transcription factor. If the "bait" and the "prey" proteins are able to interact *in vivo* to form an protein-dependent complex, the DNA-binding and activation domains of the transcription factor are brought into close proximity. This proximity allows transcription of a reporter gene (*e.g.*, LacZ), which is operably linked to a transcriptional regulatory site responsive to the transcription factor. Expression of the reporter gene can be detected, and cell colonies containing the functional transcription factor can be isolated and used to obtain the DNA sequence encoding the protein which interacts with the phospholipase-like enzyme polypeptide.

It may be desirable to immobilize either the phospholipase-like enzyme polypeptide (or polynucleotide) or the test compound to facilitate separation of bound from unbound forms of one or both of the interactants, as well as to accommodate automation of the assay. Thus, either the phospholipase-like enzyme polypeptide (or polynucleotide) or the test compound can be bound to a solid support. Suitable solid supports include, but are not limited to, glass or plastic slides, tissue culture plates, microtiter wells, tubes, silicon chips, or particles such as beads (including, but not limited to, latex, polystyrene, or glass beads). Any method known in the art can be used to attach the enzyme polypeptide (or polynucleotide) or test compound to a solid support, including use of covalent and non-

covalent linkages, passive absorption, or pairs of binding moieties attached respectively to the polypeptide (or polynucleotide) or test compound and the solid support. Test compounds are preferably bound to the solid support in an array, so that the location of individual test compounds can be tracked. Binding of a test compound to a phospholipase-like enzyme polypeptide (or polynucleotide) can be accomplished in any vessel suitable for containing the reactants. Examples of such vessels include microtiter plates, test tubes, and microcentrifuge tubes.

In one embodiment, the phospholipase-like enzyme polypeptide is a fusion protein comprising a domain that allows the phospholipase-like enzyme polypeptide to be bound to a solid support. For example, glutathione-S-transferase fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical, St. Louis, Mo.) or glutathione derivatized microtiter plates, which are then combined with the test compound or the test compound and the non-adsorbed phospholipase-like enzyme polypeptide; the mixture is then incubated under conditions conducive to complex formation (*e.g.*, at physiological conditions for salt and pH). Following incubation, the beads or microtiter plate wells are washed to remove any unbound components. Binding of the interactants can be determined either directly or indirectly, as described above. Alternatively, the complexes can be dissociated from the solid support before binding is determined.

Other techniques for immobilizing proteins or polynucleotides on a solid support also can be used in the screening assays of the invention. For example, either a phospholipase-like enzyme polypeptide (or polynucleotide) or a test compound can be immobilized utilizing conjugation of biotin and streptavidin. Biotinylated phospholipase-like enzyme polypeptides (or polynucleotides) or test compounds can be prepared from biotin-NHS(N-hydroxysuccinimide) using techniques well known in the art (*e.g.*, biotinylation kit, Pierce Chemicals, Rockford, Ill.) and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemical). Alternatively, antibodies which specifically bind to a phospholipase-like enzyme polypeptide, polynucleotide, or a test compound, but which do not interfere with a desired binding site, such as the active site of

the phospholipase-like enzyme polypeptide, can be derivatized to the wells of the plate. Unbound target or protein can be trapped in the wells by antibody conjugation.

Methods for detecting such complexes, in addition to those described above for the GST-immobilized complexes, include immunodetection of complexes using antibodies which specifically bind to the phospholipase-like enzyme polypeptide or test compound, enzyme-linked assays which rely on detecting an activity of the phospholipase-like enzyme polypeptide, and SDS gel electrophoresis under non-reducing conditions.

Screening for test compounds which bind to a phospholipase-like enzyme polypeptide or polynucleotide also can be carried out in an intact cell. Any cell which comprises a phospholipase-like enzyme polypeptide or polynucleotide can be used in a cell-based assay system. A phospholipase-like enzyme polynucleotide can be naturally occurring in the cell or can be introduced using techniques such as those described above. Binding of the test compound to a phospholipase-like enzyme polypeptide or polynucleotide is determined as described above.

Enzyme Assays

Test compounds can be tested for the ability to increase or decrease the phospholipase or esterase activity of a human phospholipase-like enzyme polypeptide. phospholipase activity can be measured, for example, as described in Example 4. _____

Enzyme assays can be carried out after contacting either a purified phospholipase-like enzyme polypeptide, a cell membrane preparation, or an intact cell with a test compound. A test compound which decreases a phospholipase or esterase activity of a phospholipase-like enzyme polypeptide by at least about 10, preferably about 50, more preferably about 75, 90, or 100% is identified as a potential therapeutic agent for decreasing phospholipase-like enzyme activity. A test compound which increases a phospholipase or esterase activity of a human phospholipase-like enzyme polypeptide by at least about 10, preferably about 50, more preferably about 75, 90, or 100% is identified as a potential therapeutic agent for increasing human phospholipase-like enzyme activity.

Gene Expression

In another embodiment, test compounds which increase or decrease phospholipase-like enzyme gene expression are identified. A phospholipase-like enzyme polynucleotide is contacted with a test compound, and the expression of an RNA or polypeptide product of the phospholipase-like enzyme polynucleotide is determined. The level of expression of appropriate mRNA or polypeptide in the presence of the test compound is compared to the level of expression of mRNA or polypeptide in the absence of the test compound. The test compound can then be identified as a modulator of expression based on this comparison. For example, when expression of mRNA or polypeptide is greater in the presence of the test compound than in its absence, the test compound is identified as a stimulator or enhancer of the mRNA or polypeptide expression. Alternatively, when expression of the mRNA or polypeptide is less in the presence of the test compound than in its absence, the test compound is identified as an inhibitor of the mRNA or polypeptide expression.

The level of phospholipase-like enzyme mRNA or polypeptide expression in the cells can be determined by methods well known in the art for detecting mRNA or polypeptide. Either qualitative or quantitative methods can be used. The presence of polypeptide products of a phospholipase-like enzyme polynucleotide can be determined, for example, using a variety of techniques known in the art, including immunochemical methods such as radioimmunoassay, Western blotting, and immunohistochemistry. Alternatively, polypeptide synthesis can be determined *in vivo*, in a cell culture, or in an *in vitro* translation system by detecting incorporation of labeled amino acids into a phospholipase-like enzyme polypeptide.

Such screening can be carried out either in a cell-free assay system or in an intact cell. Any cell which expresses a phospholipase-like enzyme polynucleotide can be used in a cell-based assay system. The phospholipase-like enzyme polynucleotide can be naturally occurring in the cell or can be introduced using techniques such as those described above. Either a primary culture or an established cell line, such as CHO or human

embryonic kidney 293 cells, can be used.

Pharmaceutical Compositions

5 The invention also provides pharmaceutical compositions which can be administered to a patient to achieve a therapeutic effect. Pharmaceutical compositions of the invention can comprise, for example, a phospholipase-like enzyme polypeptide, phospholipase-like enzyme polynucleotide, ribozymes or antisense oligonucleotides, antibodies which specifically bind to a phospholipase-like enzyme polypeptide, or mimetics, agonists, antagonists, or inhibitors of a phospholipase-like enzyme polypeptide activity. The compositions can be administered alone or in combination with at least one other agent, such as stabilizing compound, which can be administered in any sterile, biocompatible pharmaceutical carrier, including, but not limited to, saline, buffered saline, dextrose, and water. The compositions can be administered to a patient alone, or in combination with other agents, drugs or hormones.

15 In addition to the active ingredients, these pharmaceutical compositions can contain suitable pharmaceutically-acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. Pharmaceutical compositions of the invention can be administered by any number of routes including, but not limited to, oral, intravenous, intramuscular, intra-arterial, intramedullary, intrathecal, intraventricular, transdermal, subcutaneous, intraperitoneal, intranasal, parenteral, topical, sublingual, or rectal means. Pharmaceutical compositions for oral administration can be formulated using pharmaceutically acceptable carriers well known in the art in dosages suitable for oral administration. Such carriers enable the pharmaceutical compositions to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions, and the like, for ingestion by the patient.

Pharmaceutical preparations for oral use can be obtained through combination of active compounds with solid excipient, optionally grinding a resulting mixture, and

processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are carbohydrate or protein fillers, such as sugars, including lactose, sucrose, mannitol, or sorbitol; starch from corn, wheat, rice, potato, or other plants; cellulose, such as methyl cellulose, hydroxypropylmethyl-cellulose, or sodium carboxymethylcellulose; gums including arabic and tragacanth; and proteins such as gelatin and collagen. If desired, disintegrating or solubilizing agents can be added, such as the cross-linked polyvinyl pyrrolidone, agar, alginic acid, or a salt thereof, such as sodium alginate.

Dragee cores can be used in conjunction with suitable coatings, such as concentrated sugar solutions, which also can contain gum arabic, talc, polyvinylpyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments can be added to the tablets or dragee coatings for product identification or to characterize the quantity of active compound, *i.e.*, dosage.

Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a coating, such as glycerol or sorbitol. Push-fit capsules can contain active ingredients mixed with a filler or binders, such as lactose or starches, lubricants, such as talc or magnesium stearate, and, optionally, stabilizers. In soft capsules, the active compounds can be dissolved or suspended in suitable liquids, such as fatty oils, liquid, or liquid polyethylene glycol with or without stabilizers.

Pharmaceutical formulations suitable for parenteral administration can be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks' solution, Ringer's solution, or physiologically buffered saline. Aqueous injection suspensions can contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Additionally, suspensions of the active compounds can be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid

esters, such as ethyl oleate or triglycerides, or liposomes. Non-lipid polycationic amino polymers also can be used for delivery. Optionally, the suspension also can contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions. For topical or nasal administration, penetrants appropriate to the particular barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

The pharmaceutical compositions of the present invention can be manufactured in a manner that is known in the art, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping, or lyophilizing processes. The pharmaceutical composition can be provided as a salt and can be formed with many acids, including but not limited to, hydrochloric, sulfuric, acetic, lactic, tartaric, malic, succinic, etc. Salts tend to be more soluble in aqueous or other protonic solvents than are the corresponding free base forms. In other cases, the preferred preparation can be a lyophilized powder which can contain any or all of the following: 1-50 mM histidine, 0.1%-2% sucrose, and 2-7% mannitol, at a pH range of 4.5 to 5.5, that is combined with buffer prior to use.

Further details on techniques for formulation and administration can be found in the latest edition of REMINGTON'S PHARMACEUTICAL SCIENCES (Maack Publishing Co., Easton, Pa.). After pharmaceutical compositions have been prepared, they can be placed in an appropriate container and labeled for treatment of an indicated condition. Such labeling would include amount, frequency, and method of administration.

Therapeutic Indications and Methods

Cancer. Human phospholipase-like enzyme can be regulated to treat cancer. Cancer is a disease fundamentally caused by oncogenic cellular transformation. There are several hallmarks of transformed cells that distinguish them from their normal counterparts and underlie the pathophysiology of cancer. These include uncontrolled cellular

proliferation, unresponsiveness to normal death-inducing signals (immortalization), increased cellular motility and invasiveness, increased ability to recruit blood supply through induction of new blood vessel formation (angiogenesis), genetic instability, and dysregulated gene expression. Various combinations of these aberrant physiologies, along with the acquisition of drug-resistance frequently lead to an intractable disease state in which organ failure and patient death ultimately ensue.

Most standard cancer therapies target cellular proliferation and rely on the differential proliferative capacities between transformed and normal cells for their efficacy. This approach is hindered by the facts that several important normal cell types are also highly proliferative and that cancer cells frequently become resistant to these agents. Thus, the therapeutic indices for traditional anti-cancer therapies rarely exceed 2.0.

The advent of genomics-driven molecular target identification has opened up the possibility of identifying new cancer-specific targets for therapeutic intervention that will provide safer, more effective treatments for cancer patients. Thus, newly discovered tumor-associated genes and their products can be tested for their role(s) in disease and used as tools to discover and develop innovative therapies. Genes playing important roles in any of the physiological processes outlined above can be characterized as cancer targets.

Genes or gene fragments identified through genomics can readily be expressed in one or more heterologous expression systems to produce functional recombinant proteins. These proteins are characterized *in vitro* for their biochemical properties and then used as tools in high-throughput molecular screening programs to identify chemical modulators of their biochemical activities. Agonists and/or antagonists of target protein activity can be identified in this manner and subsequently tested in cellular and *in vivo* disease models for anti-cancer activity. Optimization of lead compounds with iterative testing in biological models and detailed pharmacokinetic and toxicological analyses form the basis for drug development and subsequent testing in humans.

It is known that agents which increase phospholipase A2 activity stimulate the recruitment/activation of cells of the immune system. U.S. Patent No. 5,527,775. Based

on this observation, the present invention provides methods for treating neoplastic diseases. Specifically, tumor necrosis, the killing of neoplastic cells and a reduction in the size of a neoplastic mass, can be induced in a mammal suffering from a neoplastic disease by administering an effective amount of an agent which stimulates human phospholipase-like enzyme activity or promotes arachidonic acid release, an indication of phospholipase-like enzyme activity. The stimulation of phospholipase-like enzyme activity results in the generation of eicosanoids, leading to the recruitment/activation of cell which promote an inflammatory response. The inflammatory response triggers the mammal's immune system to destroy the neoplastic cells. The response initiated by the treatment of the present invention results in a reduction in the severity of the neoplastic disease, a reduction of the tumor burden in the mammal, and a significant reduction in the number of neoplastic cells and neoplasm mass.

CNS Disorders. A_{β} -mediated neuronal cell degeneration is mediated by a biochemical cascade which requires activity of PLA2. U.S. Patent No. 5,707,821. Agents which selectively block PLA2 activity in neurons and/or glial cells and/or astrocytes or monocytes can be used to inhibit A_{β} -mediated neuronal degeneration, such as that which results from exposure of such cells to pathogenic forms of A_{β} as occurs in Alzheimer's Disease and related A_{β} -induced neurodegenerative conditions. It is likely, therefore, that inhibitors of human phospholipase-like enzyme also can be used to inhibit neuronal degeneration resulting from A_{β} toxicity.

Allergy, anaphylaxis, asthma and inflammation. It is now well-established that arachidonic acid (AA) is metabolized in mammals by two distinct pathways. U.S. Patent No. 5,070,207. The metabolism of arachidonic acid by cyclooxygenase enzymes results in the production of prostaglandins and thromboxanes. The physiological activity of the prostaglandins has already been amply elucidated in recent years. It is now known that prostaglandins arise from the endoperoxides PGG_2 and PGH_2 by the cyclooxygenase pathway of arachidonic acid metabolism. These endoperoxides are also the precursors of the thromboxanes (Tx) A_2 and B_2 . TxA_2 is a vasoconstrictor which stimulates platelet

aggregation. In the normal situation, the vasoconstrictive and platelet aggregating properties of the thromboxanes are balanced by another product arising from the endoperoxides in the cyclooxygenase pathway, prostacyclin (PGI₂), which is a vasodilator with platelet aggregation inhibitory activity. In the event prostacyclin synthesis is impaired and/or platelet activation is enhanced, then thrombosis and vasoconstriction is favored. The role of prostanoids in haemostasis and thrombosis are reviewed by Gryglewski, *CRC Crit. Rev. Biochem.* 7, 291, 1980, and Smith, *Am. J. Pathol.* 99, 743, 1980. Cyclooxygenase metabolites are known to participate directly in the inflammatory response. See Higgs *et al.*, *Annals of Clinical Research* 16, 287-99, 1984. This is through their vasodepressor activities, participation in pain and fever, augmentation of peptide mediator vascular permeability and edema forming properties. Finally, various aspects of cell mediated immunity are influenced by cyclooxygenase products.

The other pathway of AA metabolism involves lipoxygenase enzymes and results in the production of a number of oxidative products called leukotrienes. The latter are designated by the LT nomenclature system, and the most significant products of the lipoxygenase metabolic pathway are the leukotrienes B₄, C₄, and D₄. The substance denominated slow-reacting substance of anaphylaxis (SRS-A) has been shown to consist of a mixture of leukotrienes, with LTC₄ and LTD₄ as the primary products and having varying amounts of other leukotriene metabolites. See Bach *et al.*, *J. Immun.* 215, 115-18, 1980; *Biochem. Biophys. Res. Commun.* 93, 1121-26, 1980.

The significance of these leukotrienes is that a great deal of evidence has been accumulated showing that leukotrienes participate in inflammatory reactions, exhibit chemotactic activities, stimulate lysosomal enzyme release and act as important factors in the immediate hypersensitivity reaction. It has been shown that LTC₄ and LTD₄ are potent bronchoconstrictors of the human bronchi (Dahlen *et al.*, *Nature* 288, 484-486 (1980) and Piper, *Int. Arch. Appl. Immunol.* 76, suppl. 1, 43 (1985)0, which stimulate the release of mucus from airways *in vitro* [Marom *et al.*, *Am. Rev. Resp. Dis.* 126, 449 (1982)], are potent vasodilators in skin [see Bisgaard *et al.*, *Prostaglandins* 23, 797 (1982)], and

produce a wheal and flare response [Camp *et al.*, *Br. J. Pharmacol.* 80, 497 (1983)]. The nonpeptide leukotriene, LTB₄, is a powerful chemotactic factor for leukocytes [see A. W. Ford-Hutchinson, *J. Roy. Soc. Med.* 74, 831-833 (1981), which stimulates cell accumulation and affects vascular smooth muscle [see Bray, *Br. Med. Bull.* 39, 249 (1983)].

5 The activity of leukotrienes as mediators of inflammation and hypersensitivity is extensively reviewed in Bailey and Casey, *Ann. Reports Med. Chem.*, 19, 87 (1986).

Phospholipase A2 is the critical rate limiting enzyme in the arachidonic acid (AA) cascade, because it is responsible for the hydrolysis of esterified AA from the C-2 position of membrane phospholipids. This reaction generates two products: (1) free AA which is

10 then available for subsequent metabolism by either the cyclooxygenase or lipoxygenase enzymes, and (2) lysophospholipid. When alkylarachidonoyl-glycerophosphatidylcholine is acted upon by the PLA2, the generation of platelet activating factor (PAF) is initiated; PAF is pro-inflammatory in its own right [see Wedmore *et al.*, *Br. J. Pharmacol.*, 74, 916-917 (1981)]. In this regard it may be noted that the anti-inflammatory steroids are

15 thought to inhibit eicosanoid synthesis by inducing the synthesis of a PLA2 inhibitory protein denominated macrocortin, lipomodulin or lipocortin [see Flower *et al.*, *Nature, London*, 278, 456 (1979) and Hirata *et al.*, *Proc. Natn. Acad. Sci. U.S.A.*, 77, 2533 (1980)].

As the initial step leading to subsequent conversion of AA to the various eicosanoids by the cyclooxygenase and lipoxygenase pathways, the PLA2-mediated release

20 of AA from membrane phospholipids is a critical event in attempting to deal with the various physiological manifestations which are based on the activity of the eicosanoids and/or PAF. Thus, while PLA2 has been shown to be required for platelet aggregation [Pickett *et al.*, *Biochem. J.*, 160, 405 (1976)], cardiac contraction and excitation [Geisler *et al.*, *Pharm. Res. Commun.*, 9, 117 (1977)], as well as prostaglandin synthesis [Vogt, *Adv. Prostagl. Thromb. Res.*, 3, 89 (1978)], the inhibition of PLA2 is indicated in the therapeutic

25 treatment of both PAF induced or cyclooxygenase and/or lipoxygenase pathway product-mediated physiological conditions. Thus, human phospholipase-like enzyme inhibitors are a rational approach to the prevention, removal or amelioration of such

conditions as allergy, anaphylaxis, asthma and inflammation.

Allergy is a complex process in which environmental antigens induce clinically adverse reactions. The inducing antigens, called allergens, typically elicit a specific IgE response and, although in most cases the allergens themselves have little or no intrinsic toxicity, they induce pathology when the IgE response in turn elicits an IgE-dependent or T cell-dependent hypersensitivity reaction. Hypersensitivity reactions can be local or systemic and typically occur within minutes of allergen exposure in individuals who have previously been sensitized to an allergen. The hypersensitivity reaction of allergy develops when the allergen is recognized by IgE antibodies bound to specific receptors on the surface of effector cells, such as mast cells, basophils, or eosinophils, which causes the activation of the effector cells and the release of mediators that produce the acute signs and symptoms of the reactions. Allergic diseases include asthma, allergic rhinitis (hay fever), atopic dermatitis, and anaphylaxis.

Asthma is thought to arise as a result of interactions between multiple genetic and environmental factors and is characterized by three major features: 1) intermittent and reversible airway obstruction caused by bronchoconstriction, increased mucus production, and thickening of the walls of the airways that leads to a narrowing of the airways, 2) airway hyperresponsiveness caused by a decreased control of airway caliber, and 3) airway inflammation. Certain cells are critical to the inflammatory reaction of asthma and they include T cells and antigen presenting cells, B cells that produce IgE, and mast cells, basophils, eosinophils, and other cells that bind IgE. These effector cells accumulate at the site of allergic reaction in the airways and release toxic products that contribute to the acute pathology and eventually to the tissue destruction related to the disorder. Other resident cells, such as smooth muscle cells, lung epithelial cells, mucus-producing cells, and nerve cells may also be abnormal in individuals with asthma and may contribute to the pathology. While the airway obstruction of asthma, presenting clinically as an intermittent wheeze and shortness of breath, is generally the most pressing symptom of the disease requiring immediate treatment, the inflammation and tissue destruction associated with the disease

can lead to irreversible changes that eventually make asthma a chronic disabling disorder requiring long-term management.

Despite recent important advances in our understanding of the pathophysiology of asthma, the disease appears to be increasing in prevalence and severity (Gergen and Weiss, *Am. Rev. Respir. Dis.* 146, 823-24, 1992). It is estimated that 30-40% of the population suffer with atopic allergy, and 15% of children and 5% of adults in the population suffer from asthma (Gergen and Weiss, 1992). Thus, an enormous burden is placed on our health care resources. However, both diagnosis and treatment of asthma are difficult. The severity of lung tissue inflammation is not easy to measure and the symptoms of the disease are often indistinguishable from those of respiratory infections, chronic respiratory inflammatory disorders, allergic rhinitis, or other respiratory disorders. Often, the inciting allergen cannot be determined, making removal of the causative environmental agent difficult. Current pharmacological treatments suffer their own set of disadvantages. Commonly used therapeutic agents, such as beta agonists, can act as symptom relievers to transiently improve pulmonary function, but do not affect the underlying inflammation. Agents that can reduce the underlying inflammation, such as anti-inflammatory steroids, can have major drawbacks that range from immunosuppression to bone loss (Goodman and Gilman's *THE PHARMACOLOGIC BASIS OF THERAPEUTICS*, Seventh Edition, MacMillan Publishing Company, NY, USA, 1985). In addition, many of the present therapies, such as inhaled corticosteroids, are short-lasting, inconvenient to use, and must be used often on a regular basis, in some cases for life, making failure of patients to comply with the treatment a major problem and thereby reducing their effectiveness as a treatment.

Because of the problems associated with conventional therapies, alternative treatment strategies have been evaluated. Glycophorin A (Chu and Sharom, *Cell. Immunol.* 145, 223-39, 1992), cyclosporin (Alexander *et al.*, *Lancet* 339, 324-28, 1992), and a nonapeptide fragment of IL-2 (Zav'yalov *et al.*, *Immunol. Lett.* 31, 285-88, 1992) all inhibit interleukin-2 dependent T lymphocyte proliferation; however, they are known to have many other effects. For example, cyclosporin is used as a immunosuppressant after organ

transplantation. While these agents may represent alternatives to steroids in the treatment of asthmatics, they inhibit interleukin-2 dependent T lymphocyte proliferation and potentially critical immune functions associated with homeostasis. Other treatments that block the release or activity of mediators of bronchoconstriction, such as cromones or anti-leukotrienes, have recently been introduced for the treatment of mild asthma, but they are expensive and not effective in all patients and it is unclear whether they have any effect on the chronic changes associated with asthmatic inflammation. What is needed in the art is the identification of a treatment that can act in pathways critical to the development of asthma that both blocks the episodic attacks of the disorder and preferentially dampens the hyperactive allergic immune response without immunocompromising the patient.

Cardiovascular Disease. Evidence has accumulated for a potential role of PLA2 in myocardial injury to the ischemic heart. U.S. Patent No. 5,208,244. For example, it has been shown that endogenous fatty acids increase several fold in an ischemic dog heart *in vivo* and in isolated perfused ischemic rat and dog hearts. See, for example, van der Vusse et al, Circ. Res. 50, 538-46 (1982); Chien et al, Circ. Res., 54, 313-22 (1984); Burton et al, Am. J. Pathol., 124, 238-45 (1985). It has also been shown that lysophospholipids accumulate 2 to 3 fold in the ischemic cat heart *in vivo*, Corr. et al, J. Clin. Invest., 83, 927-36 (1989). The accumulation of the products of phospholipid hydrolysis implies that a PLA2 may become activated in the ischemic heart.

Lysophospholipids have also been implicated as potential mediators of sudden cardiac death, Corr et al, "Lethal Arrhythmias Resulting from Myocardial Ischemia and Infarction", Rosen & Patti, eds., Kluwer Academic Publishers, Boston, 91-014 (1989). The addition of lysophospholipids to normoxic myocardial tissue *in vitro* induces electrophysiological alterations that are similar to those observed in the ischemic heart *in vivo*. Corr et al, Circ. Res, 55, 135-54 (1984).

Most importantly, lysophospholipid accumulation in the ischemic dog heart *in vivo* has been correlated with the frequency of cardiac arrhythmias, Kinnaird et al, Lipids, 23, 32-35 (1988). Furthermore, it is known that the carnitine acyltransferase 1 inhibitor,

2-[5-(4-chlorophenyl)-pentyl]-oxirane-2-carboxylate (POCA), prevents the onset of ventricular fibrillation and ventricular tachycardia and inhibits the accumulation of lysophospholipids (and long-chain aoyloarnitines) in the ischemic cat heart *in vivo*. Corr et al, J. Clin. Invest., 83, 927-36 (1989).

5 Accelerated phospholipid catabolism by PLA2 has also been implicated as a cause of infarct damage in the ischemic heart. In the ischemic heart, ATP levels decrease. Treatment of rat neonatal myocytes with the glycolytic inhibitor iodoacetate lowers the levels of ATP which results in the release of arachidonic acid and morphological alterations of the myocytes, Chien et al, J. Clin. Invest., 75, 1770-80 (1985). One PLA2 inhibitor
10 (U26,384) prevented the release of arachidonic acid, phospholipid degradation, sarcolemmal membrane defects and the release of creatine kinase that was induced by the treatment of rat neonatal myocytes with iodoacetate, Sen et al, J. Clin. Invest., 82, 1333-38 (1988).

15 Therefore, inhibition of human phospholipase-like enzyme activity, without inhibiting other mammalian PLA2 enzymes, to prevent the accumulation of arrhythmogenic lysophospholipids in an ischemic heart is a potential therapeutic approach for the prevention of arrhythmias, infarct damage and sudden death.

Chronic obstructive pulmonary disease. Inhibitors of human phospholipase-like enzyme also can be used to treat other conditions mediated by products of the oxidation of
20 arachidonic acid, such as chronic obstructive pulmonary disease, emphysema, and acute respiratory distress syndrome. U.S. Patent No. 5,508,302. Chronic obstructive pulmonary (or airways) disease (COPD) is a condition defined physiologically as airflow obstruction that generally results from a mixture of emphysema and peripheral airway obstruction due to chronic bronchitis (Senior & Shapiro, *Pulmonary Diseases and Disorders*, 3d ed., New
25 York, McGraw-Hill, 1998, pp. 659-681, 1998; Barnes, *Chest* 117, 10S-14S, 2000). Emphysema is characterized by destruction of alveolar walls leading to abnormal enlargement of the air spaces of the lung. Chronic bronchitis is defined clinically as the presence of chronic productive cough for three months in each of two successive years. In

COPD, airflow obstruction is usually progressive and is only partially reversible. By far the most important risk factor for development of COPD is cigarette smoking, although the disease does occur in non-smokers.

Chronic inflammation of the airways is a key pathological feature of COPD (Senior & Shapiro, 1998). The inflammatory cell population comprises increased numbers of macrophages, neutrophils, and CD8⁺ lymphocytes. Inhaled irritants, such as cigarette smoke, activate macrophages which are resident in the respiratory tract, as well as epithelial cells leading to release of chemokines (e.g., interleukin-8) and other chemotactic factors. These chemotactic factors act to increase the neutrophil/monocyte trafficking from the blood into the lung tissue and airways. Neutrophils and monocytes recruited into the airways can release a variety of potentially damaging mediators such as proteolytic enzymes and reactive oxygen species. Matrix degradation and emphysema, along with airway wall thickening, surfactant dysfunction, and mucus hypersecretion, all are potential sequelae of this inflammatory response that lead to impaired airflow and gas exchange.

Obesity. Obesity, too, may be treated by regulating human phospholipase-like enzyme. Obesity and overweight are defined as an excess of body fat relative to lean body mass. An increase in caloric intake or a decrease in energy expenditure or both can bring about this imbalance leading to surplus energy being stored as fat. Obesity is associated with important medical morbidities and an increase in mortality. The causes of obesity are poorly understood and may be due to genetic factors, environmental factors or a combination of the two to cause a positive energy balance. In contrast, anorexia and cachexia are characterized by an imbalance in energy intake versus energy expenditure leading to a negative energy balance and weight loss. Agents that either increase energy expenditure and/or decrease energy intake, absorption or storage would be useful for treating obesity, overweight, and associated comorbidities. Agents that either increase energy intake and/or decrease energy expenditure or increase the amount of lean tissue would be useful for treating cachexia, anorexia and wasting disorders.

This gene, translated proteins and agents which modulate this gene or portions of

the gene or its products are useful for treating obesity, overweight, anorexia, cachexia, wasting disorders, appetite suppression, appetite enhancement, increases or decreases in satiety, modulation of body weight, and/or other eating disorders such as bulimia. Also this gene, translated proteins and agents which modulate this gene or portions of the gene or its products are useful for treating obesity/overweight-associated comorbidities including hypertension, type 2 diabetes, coronary artery disease, hyperlipidemia, stroke, gallbladder disease, gout, osteoarthritis, sleep apnea and respiratory problems, some types of cancer including endometrial, breast, prostate, and colon cancer, thrombotic disease, polycystic ovarian syndrome, reduced fertility, complications of pregnancy, menstrual irregularities, hirsutism, stress incontinence, and depression.

This invention further pertains to the use of novel agents identified by the screening assays described above. Accordingly, it is within the scope of this invention to use a test compound identified as described herein in an appropriate animal model. For example, an agent identified as described herein (*e.g.*, a modulating agent, an antisense nucleic acid molecule, a specific antibody, ribozyme, or a phospholipase-like enzyme polypeptide binding molecule) can be used in an animal model to determine the efficacy, toxicity, or side effects of treatment with such an agent. Alternatively, an agent identified as described herein can be used in an animal model to determine the mechanism of action of such an agent. Furthermore, this invention pertains to uses of novel agents identified by the above-described screening assays for treatments as described herein.

A reagent which affects phospholipase-like enzyme activity can be administered to a human cell, either *in vitro* or *in vivo*, to reduce phospholipase-like enzyme activity. The reagent preferably binds to an expression product of a human phospholipase-like enzyme gene. If the expression product is a protein, the reagent is preferably an antibody. For treatment of human cells *ex vivo*, an antibody can be added to a preparation of stem cells which have been removed from the body. The cells can then be replaced in the same or another human body, with or without clonal propagation, as is known in the art.

In one embodiment, the reagent is delivered using a liposome. Preferably, the

liposome is stable in the animal into which it has been administered for at least about 30 minutes, more preferably for at least about 1 hour, and even more preferably for at least about 24 hours. A liposome comprises a lipid composition that is capable of targeting a reagent, particularly a polynucleotide, to a particular site in an animal, such as a human. Preferably, the lipid composition of the liposome is capable of targeting to a specific organ of an animal, such as the lung, liver, spleen, heart brain, lymph nodes, and skin.

A liposome useful in the present invention comprises a lipid composition that is capable of fusing with the plasma membrane of the targeted cell to deliver its contents to the cell. Preferably, the transfection efficiency of a liposome is about 0.5 μg of DNA per 16 nmole of liposome delivered to about 10^6 cells, more preferably about 1.0 μg of DNA per 16 nmole of liposome delivered to about 10^6 cells, and even more preferably about 2.0 μg of DNA per 16 nmol of liposome delivered to about 10^6 cells. Preferably, a liposome is between about 100 and 500 nm, more preferably between about 150 and 450 nm, and even more preferably between about 200 and 400 nm in diameter.

Suitable liposomes for use in the present invention include those liposomes standardly used in, for example, gene delivery methods known to those of skill in the art. More preferred liposomes include liposomes having a polycationic lipid composition and/or liposomes having a cholesterol backbone conjugated to polyethylene glycol. Optionally, a liposome comprises a compound capable of targeting the liposome to a particular cell type, such as a cell-specific ligand exposed on the outer surface of the liposome.

Complexing a liposome with a reagent such as an antisense oligonucleotide or ribozyme can be achieved using methods which are standard in the art (see, for example, U.S. Patent 5,705,151). Preferably, from about 0.1 μg to about 10 μg of polynucleotide is combined with about 8 nmol of liposomes, more preferably from about 0.5 μg to about 5 μg of polynucleotides are combined with about 8 nmol liposomes, and even more preferably about 1.0 μg of polynucleotides is combined with about 8 nmol liposomes.

In another embodiment, antibodies can be delivered to specific tissues *in vivo* using receptor-mediated targeted delivery. Receptor-mediated DNA delivery techniques are

taught in, for example, Findeis *et al.* *Trends in Biotechnol.* 11, 202-05 (1993); Chiou *et al.*,
GENE THERAPEUTICS: METHODS AND APPLICATIONS OF DIRECT GENE TRANSFER (J.A.
Wolff, ed.) (1994); Wu & Wu, *J. Biol. Chem.* 263, 621-24 (1988); Wu *et al.*, *J. Biol. Chem.*
269, 542-46 (1994); Zenke *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 87, 3655-59 (1990); Wu *et*
5 *al.*, *J. Biol. Chem.* 266, 338-42 (1991).

Determination of a Therapeutically Effective Dose

The determination of a therapeutically effective dose is well within the capability
of those skilled in the art. A therapeutically effective dose refers to that amount of active
ingredient which increases or decreases phospholipase-like enzyme activity relative to the
10 phospholipase-like enzyme activity which occurs in the absence of the therapeutically
effective dose.

For any compound, the therapeutically effective dose can be estimated initially
either in cell culture assays or in animal models, usually mice, rabbits, dogs, or pigs. The
animal model also can be used to determine the appropriate concentration range and route
15 of administration. Such information can then be used to determine useful doses and routes
for administration in humans.

Therapeutic efficacy and toxicity, *e.g.*, ED₅₀ (the dose therapeutically effective in
50% of the population) and LD₅₀ (the dose lethal to 50% of the population), can be
determined by standard pharmaceutical procedures in cell cultures or experimental animals.
20 The dose ratio of toxic to therapeutic effects is the therapeutic index, and it can be
expressed as the ratio, LD₅₀/ED₅₀.

Pharmaceutical compositions which exhibit large therapeutic indices are preferred.
The data obtained from cell culture assays and animal studies is used in formulating a range
of dosage for human use. The dosage contained in such compositions is preferably within
25 a range of circulating concentrations that include the ED₅₀ with little or no toxicity. The
dosage varies within this range depending upon the dosage form employed, sensitivity of
the patient, and the route of administration.

The exact dosage will be determined by the practitioner, in light of factors related to the subject that requires treatment. Dosage and administration are adjusted to provide sufficient levels of the active ingredient or to maintain the desired effect. Factors which can be taken into account include the severity of the disease state, general health of the subject, age, weight, and gender of the subject, diet, time and frequency of administration, drug combination(s), reaction sensitivities, and tolerance/response to therapy. Long-acting pharmaceutical compositions can be administered every 3 to 4 days, every week, or once every two weeks depending on the half-life and clearance rate of the particular formulation.

Normal dosage amounts can vary from 0.1 to 100,000 micrograms, up to a total dose of about 1 g, depending upon the route of administration. Guidance as to particular dosages and methods of delivery is provided in the literature and generally available to practitioners in the art. Those skilled in the art will employ different formulations for nucleotides than for proteins or their inhibitors. Similarly, delivery of polynucleotides or polypeptides will be specific to particular cells, conditions, locations, etc.

If the reagent is a single-chain antibody, polynucleotides encoding the antibody can be constructed and introduced into a cell either *ex vivo* or *in vivo* using well-established techniques including, but not limited to, transferrin-polycation-mediated DNA transfer, transfection with naked or encapsulated nucleic acids, liposome-mediated cellular fusion, intracellular transportation of DNA-coated latex beads, protoplast fusion, viral infection, electroporation, "gene gun," and DEAE- or calcium phosphate-mediated transfection.

Effective *in vivo* dosages of an antibody are in the range of about 5 μ g to about 50 μ g/kg, about 50 μ g to about 5 mg/kg, about 100 μ g to about 500 μ g/kg of patient body weight, and about 200 to about 250 μ g/kg of patient body weight. For administration of polynucleotides encoding single-chain antibodies, effective *in vivo* dosages are in the range of about 100 ng to about 200 ng, 500 ng to about 50 mg, about 1 μ g to about 2 mg, about 5 μ g to about 500 μ g, and about 20 μ g to about 100 μ g of DNA.

If the expression product is mRNA, the reagent is preferably an antisense

oligonucleotide or a ribozyme. Polynucleotides which express antisense oligonucleotides or ribozymes can be introduced into cells by a variety of methods, as described above.

Preferably, a reagent reduces expression of a phospholipase-like enzyme gene or the activity of a phospholipase-like enzyme polypeptide by at least about 10, preferably about 50, more preferably about 75, 90, or 100% relative to the absence of the reagent. The effectiveness of the mechanism chosen to decrease the level of expression of a phospholipase-like enzyme gene or the activity of a phospholipase-like enzyme polypeptide can be assessed using methods well known in the art, such as hybridization of nucleotide probes to phospholipase-like enzyme-specific mRNA, quantitative RT-PCR, immunologic detection of a phospholipase-like enzyme polypeptide, or measurement of phospholipase-like enzyme activity.

In any of the embodiments described above, any of the pharmaceutical compositions of the invention can be administered in combination with other appropriate therapeutic agents. Selection of the appropriate agents for use in combination therapy can be made by one of ordinary skill in the art, according to conventional pharmaceutical principles. The combination of therapeutic agents can act synergistically to effect the treatment or prevention of the various disorders described above. Using this approach, one may be able to achieve therapeutic efficacy with lower dosages of each agent, thus reducing the potential for adverse side effects.

Any of the therapeutic methods described above can be applied to any subject in need of such therapy, including, for example, mammals such as dogs, cats, cows, horses, rabbits, monkeys, and most preferably, humans.

Diagnostic Methods

Human phospholipase-like enzyme also can be used in diagnostic assays for detecting diseases and abnormalities or susceptibility to diseases and abnormalities related to the presence of mutations in the nucleic acid sequences which encode the enzyme. For

example, differences can be determined between the cDNA or genomic sequence encoding phospholipase-like enzyme in individuals afflicted with a disease and in normal individuals. If a mutation is observed in some or all of the afflicted individuals but not in normal individuals, then the mutation is likely to be the causative agent of the disease.

5 Sequence differences between a reference gene and a gene having mutations can be revealed by the direct DNA sequencing method. In addition, cloned DNA segments can be employed as probes to detect specific DNA segments. The sensitivity of this method is greatly enhanced when combined with PCR. For example, a sequencing primer can be used with a double-stranded PCR product or a single-stranded template molecule generated by
10 a modified PCR. The sequence determination is performed by conventional procedures using radiolabeled nucleotides or by automatic sequencing procedures using fluorescent tags.

Genetic testing based on DNA sequence differences can be carried out by detection of alteration in electrophoretic mobility of DNA fragments in gels with or without
15 denaturing agents. Small sequence deletions and insertions can be visualized, for example, by high resolution gel electrophoresis. DNA fragments of different sequences can be distinguished on denaturing formamide gradient gels in which the mobilities of different DNA fragments are retarded in the gel at different positions according to their specific melting or partial melting temperatures (*see, e.g., Myers et al., Science 230, 1242, 1985*).
20 Sequence changes at specific locations can also be revealed by nuclease protection assays, such as RNase and S 1 protection or the chemical cleavage method (*e.g., Cotton et al., Proc. Natl. Acad. Sci. USA 85, 4397-4401, 1985*). Thus, the detection of a specific DNA sequence can be performed by methods such as hybridization, RNase protection, chemical cleavage, direct DNA sequencing or the use of restriction enzymes and Southern blotting
25 of genomic DNA. In addition to direct methods such as gel-electrophoresis and DNA sequencing, mutations can also be detected by *in situ* analysis.

Altered levels of a phospholipase-like enzyme also can be detected in various tissues. Assays used to detect levels of the receptor polypeptides in a body sample, such

as blood or a tissue biopsy, derived from a host are well known to those of skill in the art and include radioimmunoassays, competitive binding assays, Western blot analysis, and ELISA assays.

5 All patents and patent applications cited in this disclosure are expressly incorporated herein by reference. The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific examples which are provided for purposes of illustration only and are not intended to limit the scope of the invention.

10 **EXAMPLE 1**

Expression of recombinant human phospholipase-like enzyme

The *Pichia pastoris* expression vector pPICZB (Invitrogen, San Diego, CA) is used to produce large quantities of recombinant human phospholipase-like polypeptides in yeast. 15 The phospholipase-like enzyme-encoding DNA sequence is derived from SEQ ID NO:1. Before insertion into vector pPICZB, the DNA sequence is modified by well known methods in such a way that it contains at its 5'-end an initiation codon and at its 3'-end an enterokinase cleavage site, a His6 reporter tag and a termination codon. Moreover, at both termini recognition sequences for restriction endonucleases are added and after digestion 20 of the multiple cloning site of pPICZ B with the corresponding restriction enzymes the modified DNA sequence is ligated into pPICZB. This expression vector is designed for inducible expression in *Pichia pastoris*, driven by a yeast promoter. The resulting pPICZ/md-His6 vector is used to transform the yeast.

25 The yeast is cultivated under usual conditions in 5 liter shake flasks and the recombinantly produced protein isolated from the culture by affinity chromatography (Ni-NTA-Resin) in the presence of 8 M urea. The bound polypeptide is eluted with buffer, pH 3.5, and neutralized. Separation of the polypeptide from the His6 reporter tag is

accomplished by site-specific proteolysis using enterokinase (Invitrogen, San Diego, CA) according to manufacturer's instructions. Purified human phospholipase-like enzyme polypeptide is obtained.

5 **EXAMPLE 2**

Identification of test compounds that bind to phospholipase-like enzyme polypeptides

Purified phospholipase-like enzyme polypeptides comprising a glutathione-S-transferase protein and absorbed onto glutathione-derivatized wells of 96-well microtiter plates are contacted with test compounds from a small molecule library at pH 7.0 in a physiological buffer solution. Human phospholipase-like enzyme polypeptides comprise the amino acid sequence shown in SEQ ID NO:2. The test compounds comprise a fluorescent tag. The samples are incubated for 5 minutes to one hour. Control samples are incubated in the absence of a test compound.

The buffer solution containing the test compounds is washed from the wells. Binding of a test compound to a phospholipase-like enzyme polypeptide is detected by fluorescence measurements of the contents of the wells. A test compound which increases the fluorescence in a well by at least 15% relative to fluorescence of a well in which a test compound is not incubated is identified as a compound which binds to a phospholipase-like enzyme polypeptide.

20

EXAMPLE 3

Identification of a test compound which decreases phospholipase-like enzyme gene expression

A test compound is administered to a culture of human cells transfected with a phospholipase-like enzyme expression construct and incubated at 37 °C for 10 to 45 minutes. A culture of the same type of cells which have not been transfected is incubated

for the same time without the test compound to provide a negative control.

RNA is isolated from the two cultures as described in Chirgwin *et al.*, *Biochem. 18*, 5294-99, 1979). Northern blots are prepared using 20 to 30 μ g total RNA and hybridized with a 32 P-labeled phospholipase-like enzyme-specific probe at 65 °C in Express-hyb (CLONTECH). The probe comprises at least 11 contiguous nucleotides selected from the complement of SEQ ID NO:2. A test compound which decreases the phospholipase-like enzyme-specific signal relative to the signal obtained in the absence of the test compound is identified as an inhibitor of phospholipase-like enzyme gene expression.

EXAMPLE 4

Demonstration of human phospholipase-like enzyme activity

Human phospholipase-like enzyme activity can be demonstrated by an *in vitro* hydrolysis assay with vesicles containing 1-palmitoyl-2-[1- 14 C]oleoyl phosphatidylcholine (Sigma Chemical Co.). Phospholipase activity is demonstrated by analysis of the cleavage products isolated from the hydrolysis reaction mixture.

Vesicles containing 1-palmitoyl-2-[1- 14 C]oleoyl phosphatidylcholine (Amersham Corp.) are prepared by mixing 2.0 μ Ci of the radiolabeled phospholipid with 12.5 mg of unlabeled 1-palmitoyl-2-oleoyl phosphatidylcholine and drying the mixture under N_2 . Two and a half ml of 150 mM Tris-HCl, pH 7.5, is added, and the mixture is sonicated and centrifuged. The supernatant may be stored at 4 °C. The final reaction mixture contains 0.25 ml of Hanks buffered salt solution supplemented with 2.0 mM taurochenodeoxycholate, 1.0% bovine serum albumin, 1.0 mM $CaCl_2$, pH 7.4, 150 μ g of 1-palmitoyl-2-[1- 14 C]oleoyl phosphatidylcholine vesicles, and various amount of human phospholipase-like enzyme diluted in PBS. After incubation for 30 min at 37 °C, 20 μ g each of lyso-phosphatidylcholine and oleic acid are added as carriers and each sample is extracted for total lipids.

The lipids are separated by thin layer chromatography, using a two solvent system

of chloroform:methanol:acetic acid:water (65:35:8:4) until the solvent front is halfway up the plate, the process is then continued with hexane:ether:acetic acid (86:16:1) until the solvent front is at the top of the plate. The lipid-containing areas are visualized with I2 vapor; the spots are scraped, and their radioactivity is determined by scintillation counting.

The amount of radioactivity released as fatty acids will increase as a greater amount of

Human phospholipase-like enzyme is added to the assay mixture while the amount of radioactivity released as lyso-phosphatidylcholine will remain low. This demonstrates that human phospholipase-like enzyme cleaves at the sn-2 and not the sn-1 position, exhibiting phospholipase A2 activity.

EXAMPLE 5

Identification of a test compound which decreases phospholipase-like enzyme activity

A test compound is administered to a culture of human cells transfected with a phospholipase-like enzyme expression construct and incubated at 37 °C for 10 to 45 minutes. A culture of the same type of cells which have not been transfected is incubated for the same time without the test compound to provide a negative control.

A test compound which decreases the phospholipase activity of the phospholipase-like enzyme relative to the phospholipase activity in the absence of the test compound is identified as an inhibitor of phospholipase-like enzyme activity.

EXAMPLE 6

Identification of molecules which interact with human phospholipase-like enzyme

Human phospholipase-like enzyme or biologically active fragments thereof are labeled with ¹²⁵I Bolton-Hunter reagent (Bolton *et al.*, *Biochem. J.* 133, 529, 1973).

Candidate molecules previously arrayed in the wells of a multi-well plate are incubated with the labeled human phospholipase-like enzyme, washed, and any wells with labeled

human phospholipase-like enzyme complex are assayed. Data obtained using different concentrations of human phospholipase-like enzyme are used to calculate values for the number, affinity, and association of human phospholipase-like enzyme with the candidate molecules.

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